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Extraction of proteins other than myosin from the isolated rabbit myofibril. By S. V. Perry and A. Corsi

Some observations on the localization of myosin, actin and tropomyosin in the rabbit myofibril. By A. Corsi and S. V. Perry

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An assay of insulin by fibril formation from small samples of pancreas. By G. M. GRODSKY

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CORRIGENDUM

Determination of creatine in biological fluids

By D. R. Anderson, C. M. Williams, G. M. Krise and R. M. Dowben

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p. 261, col. 1, last line but one of Results section:

for **2·9±1·5 mg./ml.** ***read*** **2·9±1·5 μg./ml.**

Guanosine Di- and Tri-phosphates in the Phosphate Metabolism of Cerebral Tissues Promoted by Electrical Pulses

By P. J. HEALD

Department of Biochemistry, Institute of Psychiatry (British Postgraduate Medical Federation, University of London), Maudsley Hospital, London, S.E. 5

(*Received* 2 *May* 1957)

In a previous communication (Heald, 1957*b*) it was shown that the 'phosphoprotein' fraction of cerebral tissues was implicated in the rapid phosphate-exchange reactions promoted by applied electrical pulses (Heald, 1956*b*). It was pointed out that owing to the crudeness of the analytical methods employed the nature of the phosphoprotein could not be determined, more especially since its estimation depended upon a preliminary degradation to inorganic phosphate. It was also considered that since the phospholipid fraction showed no change in specific radioactivity upon the passage of pulses, the phospholipids were not involved in the rapid-exchange reactions which were described. However, it was pointed out by Dr G. H. Sloane-Stanley and subsequently by Professor J. Folch that treatment of the tissue with trichloroacetic acid to remove phosphates soluble in this extractant before further fractionation would affect the extractability of the phospholipids, some of which might conceivably remain unextracted and be degraded to inorganic phosphate during the analysis for 'phosphoprotein'.

For these reasons attempts were made to isolate from cerebral tissues, under conditions less degradative than those previously used, a phosphate fraction or fractions which increased in specific radioactivity when electrical pulses were applied for 10 sec. to tissue which had metabolized radioactive phosphate for a brief period. Such an increase in specific radioactivity under these conditions had previously been found to occur with the phosphoprotein fraction.

This has led to the discovery that guanosine di- and tri-phosphates are components of cerebral tissue implicated in this type of response. A preliminary account of this work was presented to the Biochemical Society in November 1956 (Heald, 1957*a*).

MATERIALS AND METHODS

Tissues, media and general methods of analysis and apparatus

These have been described in detail in previous publications (Heald, 1956*a*, *b*, 1957*b*) together with techniques of manipulation of the cerebral slices. Only procedures specific to the work described here are given.

Fixation and extraction of cerebral slices

Slices were fixed either in 5 ml. of $CHCl_3$–methanol (2:1, v/v) (Folch, Ascoli, Lees, Meath & LeBaron, 1951), at $-15°$, or in saturated $(NH_4)_2SO_4$ at 0° buffered to pH 4 with 0·1 M-sodium acetate (Lowry & Lopez, 1946). They were disintegrated in small glass tubes with a fitting pestle and centrifuged at 25 000 *g* in the superspeed head of the M.S.E. Major centrifuge for 15 min. The temperatures of centrifuging were $-15°$ for $CHCl_3$–methanol extracts and 0° for $(NH_4)_2SO_4$ extracts.

Tissues fixed in ammonium sulphate. The residues remaining after extraction were digested for the determination of total phosphate and radioactivity. The $(NH_4)_2SO_4$ supernatants were analysed for inorganic phosphate as described by Lowry & Lopez (1946) with the addition of $CuSO_4$ to a final concentration of 4×10^{-3} M to increase the rate of reaction (Peel, Fox & Elsden, 1955). Phosphocreatine was determined in these extracts as the difference between inorganic phosphate and the total phosphates extractable into *iso*butanol after incubation of an extract diluted five times with acid molybdate for 30 min. at 25°. Dilution of the extracts was essential, for above 20% saturation with $(NH_4)_2SO_4$ the phosphomolybdate complex was not extracted by *iso*butanol.

Tissues fixed in chloroform–methanol. After extraction with at least 20 vol. of $CHCl_3$–methanol (2:1, v/v) the residues were washed once with ethanol at $-15°$, and extracted at 0° four or five times with 0·5 M-2-amino-2-hydroxymethylpropane-1:3-diol (tris) saturated at pH 7·4 with $(NH_4)_2SO_4$. The ethanol wash was essential to remove $CHCl_3$ from the residue, which otherwise formed an unmanageable gum when $(NH_4)_2SO_4$ was added. The residues were extracted three times with aq. NH_3 soln. (0·28 M), pH 10·5–11·0, at 0°, a fluid:tissue ratio of 0·5 ml.:100 mg. original wet wt. of tissue being used. This brought the bulk of the radioactive material into solution. The radioactive phosphates in this extract were further fractionated upon a column of Dowex-1 chloride either 4 or 8% cross-linked.

Treatment of the chloroform–methanol extracts

To remove contaminating water-soluble phosphates the extracts were washed first with 0·2 vol. of 0·03 M-NaCl and then five times with 0·2 vol. of the upper aqueous phase of an equilibrated mixture of 5 parts of $CHCl_3$–methanol (2:1, v/v) and 1 part of 0·03 M-NaCl. This mixture was considered to correspond to that produced by the addition of 0·2 vol. of NaCl solution to the original extracts. Unless NaCl is included the aqueous layers contain acidic phospholipids and sulphatides and in addition tend to form unmanageable

gels. The addition of NaCl prevents this (Folch, Lees & Sloane-Stanley, 1954). After washing, samples of the $CHCl_3$ extract were taken for the determination of total phosphorus and radioactivity.

Fractionation of the ammoniacal extract on Dowex-1 chloride

For experiments involving 800 mg. of tissue, a column 1 cm.^{2} × 3 cm. was used. The ammoniacal extract was diluted 10–20 times with water, to reduce the concentration of $(NH_4)_2SO_4$ to below 0·01 M, and was passed through the resin at the rate of 3 ml./min. Usually all the radioactivity was absorbed. If counts appeared in the effluent from such a column it was attributed to insufficient dilution. When all the extract had been added the column was washed with 50–100 ml. of water and eluted with 0·005 N-HCl, 0·01 N-HCl and 0·1 N-HCl containing 0·5 M-NH_4Cl. Two fractions *A* and *B* were obtained (Fig. 1*a*). The choice of eluents was made on the basis of preliminary experiments with the gradient-elution technique with differing initial concentrations of HCl and NH_4Cl.

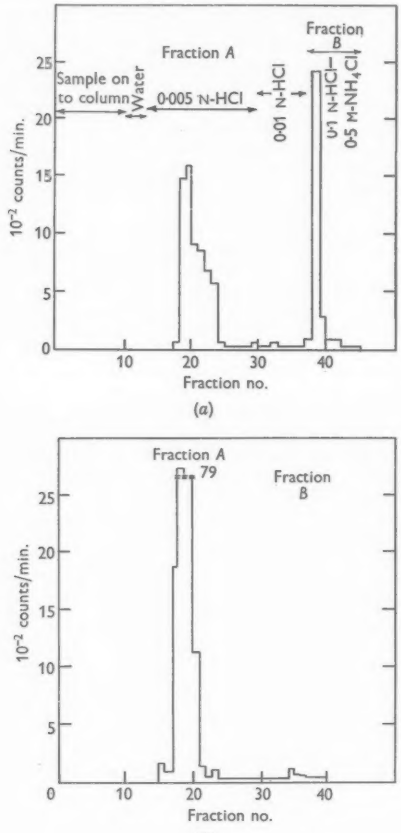

Fig. 1. Fractionation of radioactive phosphates in ammoniacal extracts of cerebral tissues upon Dowex-1 chloride. Column 1 cm.^{2} × 3 cm. Fractions *A* and *B* were eluted with 0·005 N-HCl and 0·1 N-HCl containing 0·5 M-NH_4Cl, respectively. (*a*) Fractionation of an extract prepared from tissue which had metabolized radioactive phosphate; (*b*) fractionation of an inactive extract to which radioactive orthophosphate had been added. Fraction volumes were 10 ml. in all cases.

Analysis of fractions *A* and *B* for total phosphate and radioactivity was accomplished by evaporating almost to dryness and digesting the residues as described previously (Heald, 1956*a*). The residue of fraction *B* was first heated to remove excess of NH_4Cl since high concentrations of this interfere with the extraction of the phosphomolybdate complex.

Purification of fraction B. Further purification of fraction *B* was achieved by adsorption on to acid-washed charcoal (British Drug Houses Ltd.) and elution with aq. 0·01 M-NH_3 soln.–0·1 M-phenol. Before elution, the column was washed with water until the effluent no longer gave any reaction with barium acetate solution. Phenol was removed from the eluate by extraction with ether and the aqueous solution was freeze-dried. The products, fractions *C*, were pale straw-coloured powders, free from chloride and sulphate.

The whole procedure is outlined in Fig. 2.

Preparation of fraction C *from ox brain*

Ox brain was obtained from an abbatoir (Croydon Abbatoir, 33 Cuthbert Road, Croydon), and was removed whole and placed in crushed solid CO_2 within 3–4 min. of the death of the animal. This was found to be necessary since the quantity of the fraction sought decreased to one-fifth of the level found in frozen brain if the tissue was allowed to remain at air temperature during the 1–1·5 hr. required for transport to the laboratory. In a typical experiment the brain (320 g.) was broken while frozen into portions of 38 g. and each portion was extracted in a Waring Blendor with 760 ml. of ice-cold $CHCl_3$–methanol (2:1, v/v) for 3 min. The suspension was filtered and the residue washed on the filter with 200 ml. of ethanol at −15°. The washed residue was extracted in the Blendor with saturated $(NH_4)_2SO_4$ (pH 7·4, 0·5 M-tris), 200 ml. of solution being used/10 g. of residue. This procedure resulted in some rise in temperature and required speed of operation. The suspension was filtered at +2°, this being simpler than centrifuging from such a dense medium. The residue was then extracted in the Blendor with aq. 0·28 M-NH_3 soln., 200 ml./10 g. of residue. The extracts were centrifuged, combined and diluted to 10 l. To this was added, as a marker, a radioactive extract prepared from guinea-pig cerebral tissue which had been allowed to metabolize ^{32}P under the conditions used in this and previous studies (Heald, 1956*b*). The diluted total extract was divided into two portions and each was passed, under pressure, through a column of Dowex-1 chloride 1·8 cm.^{2} × 15 cm. This operation was conducted in the cold room to minimize breakdown of labile phosphates during the long period necessary to pass the solutions through the resins. Dividing the extract as above enabled the whole 10 l. to be passed through overnight. Fraction *B* was then eluted as described above, the radioactivity of the marker

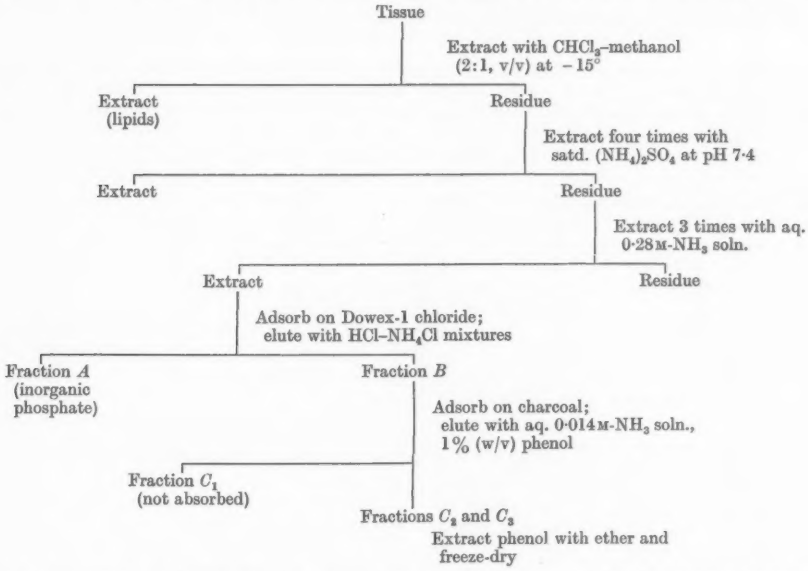
Tissue
Extract with $CHCl_3$–methanol (2:1, v/v) at −15°
Extract (lipids)
Residue
Extract four times with satd. $(NH_4)_2SO_4$ at pH 7·4
Extract
Residue
Extract 3 times with aq. 0·28 M-NH_3 soln.
Extract
Residue
Adsorb on Dowex-1 chloride; elute with HCl–NH_4Cl mixtures
Fraction A (inorganic phosphate)
Fraction B
Adsorb on charcoal; elute with aq. 0·014 M-NH_3 soln., 1% (w/v) phenol
Fraction C_1 (not absorbed)
Fractions C_2 and C_3
Extract phenol with ether and freeze-dry

Fig. 2. Fractionation of cerebral tissue to obtain phosphates increasing in specific radioactivity when derived from slices metabolizing radioactive phosphate and subjected to electrical pulses.

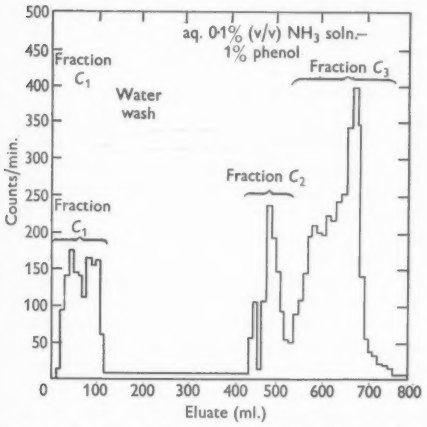

Fig. 3. Separation of three fractions from fraction *B* (see Figs. 1 and 2) by adsorption on charcoal and elution with aq. 0·014 M-NH_3 soln., 1% (w/v) phenol. Fraction C_1 was not adsorbed on the column. The column consisted of acid-washed charcoal, 1·8 cm.² × 15 cm., 10 ml. fractions being collected.

sample indicating the point at which the fraction was removed from the column.

Fraction *B* was refractionated on charcoal columns 1·8 cm.² × 15 cm. containing about 10 g. of charcoal. A typical fractionation is shown in Fig. 3. Two major fractions were usually obtained, designated C_2 and C_3 respectively, fraction C_1 being that not adsorbed on charcoal. After removal of phenol, the eluates were freeze-dried.

Analytical methods

Protein. This was determined by the method of Lowry, Rosebrough, Farr & Randall (1951), with a 1:250 dilution of serum as an arbitrary standard.

Pentose. This was estimated according to the procedure of Albaum & Umbreit (1947), readings of the colour intensity being taken at 7, 20 and 45 min. respectively to distinguish between the pentose 3′- and 5′-phosphates.

Paper chromatography. This method was used for the separation of guanosine and adenosine di- and tri-phosphates. The solvent was propan-1-ol–aq. NH_3 soln.–water (60:30:30, by vol.). In this solvent adenyl derivatives run ahead of guanyl derivatives and the two are separable in 18–24 hr. Purines were separated from hydrolysates of the nucleotides (1 hr. in N-HCl at 100°) by chromatography in propan-2-ol–HCl (Wyatt, 1951). They were detected in ultraviolet light from a Chromatolite lamp (Hanovia Ltd., Slough, Bucks) and eluted in 0·1 N-HCl for determination of the absorption spectra.

Paper electrophoresis. Nucleoside di- and tri-phosphates were separated by paper electrophoresis in 0·05 M-citrate buffer, pH 3·5.

RESULTS

The choice of conditions for extraction was determined by the criterion that the specific radioactivity of the fractions examined increased when these were

Table 1. *Effect of electrical pulses on the distribution of radioactive phosphorus in fractions of cerebral tissue initially denatured in chloroform–methanol (2:1, v/v) at −15°*

After denaturation the tissues were extracted with saturated $(NH_4)_2SO_4$ at pH 7·4. Conditions as in the Methods section. Values given are the specific radioactivities of the phosphorus of each fraction. Different amounts of radioactive phosphorus were used in different experiments. Pulses, condenser pulses 18 v peak potential, 0·4 msec. duration, were applied for 10 sec. only.

	$CHCl_3$–methanol extract			Residue after $(NH_4)_2SO_4$ extraction		
Expt. no.	With pulses	Without pulses	Difference	With pulses	Without pulses	Difference
1	0·52	0·63	−0·11	5·40	3·00	+ 2·40
2	0·04	0·04	0·0	5·85	3·16	+ 2·69
3	0·28	0·30	−0·02	17·4	9·65	+ 7·75
4	3·12	1·40	+1·72	43·5	38·0	+ 5·50
5	4·70	2·54	+2·16	59·0	46·0	+13·0
6	0·82	0·45	+0·37	5·25	4·4	+ 0·85
7	0·69	0·62	+0·07	25·6	24·5	+ 1·10
8	1·53	1·44	+0·09	42·0	32·7	+ 9·3
9	—	—	—	26·4	14·3	+12·1
	$P<0·2>0·1$			$P<0·01$		

derived from cerebral slices incubated in the presence of radioactive phosphorus and subjected to electrical pulses. It was also required that such fractions were free from contaminating inorganic phosphate, phosphocreatine and adenosine triphosphate.

Experiments in which the tissue was fixed in ice-cold saturated $(NH_4)_2SO_4$, pH 4 (Lowry & Lopez, 1946), failed to demonstrate that such a change in specific radioactivity occurred in the tissue residues. It was found that if the phospholipids were removed by initially fixing the slice in $CHCl_3$–methanol (2:1, v/v) (Folch *et al.* 1951) and subsequently washing the residue with saturated $(NH_4)_2SO_4$ (pH 7·4) at 0°, the washed residue increased in specific radioactivity if pulses had been passed through the original tissue slice (Table 1). No significant increase was noted in the activity of the phospholipid fraction.

Determination of phosphocreatine and inorganic phosphate in the extracts showed that they were quantitatively removed from the tissue. Contaminating radioactive inorganic phosphate and other water-soluble phosphates were removed from the $CHCl_3$–methanol extract by washing as described in the Methods section. This reduced contamination by added inorganic phosphate to 0·1 %. Similarly, extraction of the non-phospholipid residue with saturated $(NH_4)_2SO_4$ reduced contamination to 0·3–0·5 % of the amount of orthophosphate originally added. Further extractions did not reduce the degree of contamination any more and this constituted an appreciable error if quantities in excess of 4–5 μc of ^{32}P were added to each tissue slice. Contamination from this quantity was calculated to alter the specific radioactivity of the final residue by 0·3–0·5 unit only. The third and fourth $(NH_4)_2SO_4$ extracts did not absorb at 260 mμ, indicating that all extractable nucleotides had been removed.

Adenosine triphosphatase activity of the tissue was absent during fixation. Thus for tissue fixed in $CHCl_3$–methanol in the presence of 0·771 μmole of adenosine triphosphate the levels of inorganic phosphate were $0·17 \pm 0·02$ (6) μmole of P/100 mg. of slice, and for tissues fixed in the absence of adenosine triphosphate, inorganic phosphate levels were $0·13 \pm 0·02$ (4) μmole of P/100 mg. of slice. No activity was detectable in the tissue residue after extraction of the phospholipids.

Extraction of the non-phospholipid residue

Since previous work (Heald, 1957*b*) had suggested that the required fraction was a 'phosphoprotein', most use was made of the extraction at alkaline pH. In Table 2 is shown the percentage activity extracted from the residue by solutions of different pH. As the pH of the extractant increased, the percentage of radioactivity in the extract increased. Above pH 6·8 the quantity of protein extracted paralleled the radioactivity of the extract (Table 2, column 3), and it was therefore considered that the same material was being extracted in increasing quantity. Since the material sought was thought to be labile in alkali, solutions above pH 11·5 were not used. Ammonia solution was the extractant of choice since it could be easily removed.

The specific radioactivity of the ammoniacal extract increased when pulses were applied to the tissue slices. Inorganic phosphate, as estimated by the method of Berenblum & Chain (1938), also increased in specific radioactivity. No significant change was noted in the extracted residue (Table 3). The increase in the specific radioactivity of the inorganic phosphate fraction was contrary to that expected from previous results (Heald, 1956*b*) and

proved very difficult to assess for the following reasons. Direct application of the Berenblum & Chain technique to the ammoniacal extracts, containing added radioactive inorganic phosphate, failed to extract all the added phosphate. This was due to the formation of an opaque layer at the interphase of the *iso*butanol and the acidified extract. This layer contained neither radioactivity nor phosphorus until molybdate was added to the acid solution, when the layer became radioactive. Presumably the phosphomolybdate complex was partially adsorbed by the layer. Inclusion of this layer with the *iso*butanol extracts led to correct values for radioactivity but gave high values for inorganic phosphate. Thus in these experiments the levels of inorganic phosphate in solution were, by the Berenblum & Chain procedure, $1{\cdot}07 \pm 0{\cdot}15$ (3) μmole/g. wet wt. of original tissue, and by the Lowry & Lopez (1946) method $0{\cdot}64 \pm 0{\cdot}12$ (3) μmole/g. wet wt. The Lowry–Lopez method is generally considered to yield a correct value for inorganic phosphate in solution. The quantity of inorganic phosphate found could not arise by contamination or adsorption of the phosphate of the tissue slice (normally 1·5–2·0 μmoles/g. wet wt.), since such contamination would not exceed 0·5 % (see above).

Attempts to separate inorganic phosphate and other phosphates in the extracts, by freeze-drying and subjecting the residue to paper electrophoresis, were unsuccessful. All the fractions obtained were shown by control experiments to contain inorganic phosphate as a contaminant. Trichloroacetic acid at a final concentration of 10 % (w/v) did not precipitate any radioactive component.

It was found possible to separate inorganic phosphate and a metabolically active phosphate fraction by chromatography on Dowex-1 chloride. A typical fractionation of extracts from 1 to 2 g. of guinea-pig cerebral slices is shown in Fig. 1 (*a*), and in Fig. 1 (*b*) is shown the fractionation of an inactive extract to which radioactive inorganic phosphate had been added. Clearly the fraction *B* did not contain inorganic phosphate. Fraction *A* was not always retained by the resin, particularly if the quantity of ammonia in the extracts was increased. Thus continued washing with aq. 0·02–0·03 M-NH_3 soln., such as occurred if much extract was to be fractionated, brought most of fraction *A* off the column before elution with HCl was begun. Possibly inorganic phosphate was bound to some basic protein or peptide in the mixture.

Fractions *A* and *B* together accounted for 80–90 % of the total radioactivity added to the column, less than 3 % being retained after elution of fraction *B*. In terms of phosphorus these fractions accounted for only 50 % of the total phosphorus present in the extract. Thus the ammoniacal extracts contained $2{\cdot}21 \pm 0{\cdot}17$ (12) μmoles of P/g. wet wt. of tissue whereas fractions *A* and *B* contained $0{\cdot}56 \pm 0{\cdot}1$ (9) and $0{\cdot}68 \pm 0{\cdot}12$ (8) μmole of P/g. wet wt. respectively. The discrepancy is presumably due to inactive phosphates or phosphates of low radioactivity which were not eluted by 0·1 N-HCl, 0·5 M-NH_4Cl.

Table 2. *Extraction of radioactive phosphate material and protein from the non-phospholipid residue of cerebral tissue by various extractants*

Residues were shaken with extractant, 0·5 ml. of extractant/100 mg. original wet wt. of tissue being used, at 0–2° for periods of 5 min. Extractions were repeated until little more radioactivity appeared in the extracts.

Extractant	pH	Counts in solution as percentage of the counts originally present	Protein extracted as percentage of the total originally present	Ratio: $\frac{\%\ \text{protein}}{\%\ \text{counts}}$
Acetate (0·05 M)	4·0	32·0 ± 6·0 (3)	3·61 ± 0·8 (3)	0·129
Tris (0·05 M)	6·8	41·0 ± 4·4 (3)	10·8 ± 1·1 (3)	0·263
Tris (0·05 M)	9·0	57·1 ± 4·4 (3)	13·5 ± 0·6 (3)	0·24
Ammonia soln. (0·28 M)	11·5	78·5 ± 6·0 (6)	19·9 ± 2·0 (3)	0·258

Table 3. *Effects of electrical pulses on the distribution of radioactivity in ammoniacal extracts of guinea-pig cerebral slices, after removal of phospholipids*

Conditions of incubation of tissue were as given in the Methods section. Pulses, 18 v peak potential, 0·4 msec. duration, were applied for 10 sec. Values are specific radioactivities (counts/min./μg. of P) ± S.E.M. Figures in parentheses are the number of determinations.

Fraction					
Total P in ammonia extract		Orthophosphate in extract		Residual P	
With pulses	Without pulses	With pulses	Without pulses	With pulses	Without pulses
45·1 ± 3·6 (3)	18·0 ± 1·97 (3)	56·7 ± 4·0 (3)	29·4 ± 5·5 (3)	8·28 ± 1·14 (3)	6·47 ± 0·84 (3)

Table 4. *Effect of electrical pulses on the specific radioactivity of two phosphate fractions obtained from cerebral slices after incubation in saline containing radioactive inorganic phosphate*

Conditions were as described in the Methods section. Fractions *A* and *B* refer to the fractions depicted in Fig. 1*a*. Differing amounts of radioactivity were used in different experiments. Condenser pulses 18 v peak potential, 0·4 msec. duration, were applied for 10 sec.

	Specific radioactivity of the fraction				
	Fraction *A*		Fraction *B*		
Expt. no.	With pulses	Without pulses	With pulses	Without pulses	Difference
1	565	692	178	118	+60
2	547	517	112	88	+24
3	852	745	226	80	+146
4	1120	960	196	90	+106
					$P<0{\cdot}001$

The effect of electrical pulses upon the specific radioactivity of fractions *A* and *B* is shown in Table 4. When applied for a period of 10 sec., pulses induced an increase averaging 95 % in the activity of fraction *B* and a smaller but variable increase in fraction *A*. The difference between the individual experiments of Table 4 is probably due to the different quantities of radioactive phosphate used.

That the phosphate in fraction *B* was metabolically incorporated was shown by two types of experiment. In the first, radioactive phosphate was added to slices metabolizing in a glucose medium, 10 sec. before fixation. During this period pulses were passed. Fractionation on Dowex-1 revealed a pattern similar to that of Fig. 1 (*b*). This experiment would appear to rule out the increase in activity being due to permeability changes induced by applied pulses. In the second experiment glucose was omitted from the medium, but otherwise the experiment was performed in a manner identical with that yielding the results in Fig. 1 (*a*). Here also pulses were applied during the last 10 sec. before fixation. The specific radioactivity of fraction *B* was 0·17–0·20 of that obtained in experiments where glucose was present.

Removal of salts from fraction B. As obtained from the Dowex column fraction *B* contained a high concentration of NH_4Cl and $(NH_4)_2SO_4$. These were removed by adsorbing the fraction on charcoal and eluting with phenol–aq. NH_3 soln. (see Fig. 3). A small amount (12 %) was not adsorbed and about 4 % was retained by the column. Recovery of phosphorus was almost quantitative. Thus in three determinations the amounts added to the column were 0·60, 0·44 and 0·5 μmole of P/g. wet wt., and the amounts recovered were: (*a*) not absorbed, 0·003, 0·02 and 0·02 μmole of P/g. wet wt.; (*b*) as the major peak, 0·57, 0·36 and 0·42 μmole of P/g. wet wt. respectively.

Identification of the components of fraction B

Ox brain was obtained and fractionated as described in the Methods section. The peaks labelled C_2 and C_3 (see Fig. 3) were examined separately. Paper chromatography in propan-1-ol–aq. NH_3 soln.–water revealed that each fraction contained at least two components designated $C_{2(1)}$, $C_{2(2)}$ and $C_{3(1)}$, $C_{3(2)}$, for the slowest- and fastest-running components respectively. From the positions of the spots on guide strips suitable sections of larger chromatograms were cut out and eluted with water. Each eluate upon paper electrophoresis at pH 3·5 was further resolved into two components, $C_{2(1),\,a,\,b,}$ etc., subscripts *a* and *b* representing the slower- and faster-moving components respectively.

Fractions $C_{2(1)}$ and $C_{3(1)}$ contained only guanine, whereas fraction $C_{3(2)}$ contained only adenine. Fraction $C_{2(2)}$ contained adenine and guanine. Fractions $C_{2(2)\,a,\,b,}$ were lost after electrophoresis but, from the position of the marker spots and subsequent analysis of the other fractions, were considered to be adenosine diphposhate and guanosine triphosphate respectively.

The time course of development of orcinol colour during the determination of pentose was identical with that of an adenosine 5′-phosphate (Albaum & Umbreit, 1947). This analysis was carried out either on the single components or, as in fraction $C_{2(2)}$, upon the mixture. The absorption spectra of the orcinol colours were identical with that obtained from adenosine 5′-triphosphate, indicating that hexoses were absent. It is considered that the components were pentose 5′-phosphates, the pentose probably being ribose. Analysis of the individual phosphates and suggested identity is given in Table 5.

In Table 6 is shown the effect of pulses upon the distribution of radioactivity in the main fractions described above. Clearly the increase in specific radioactivity took place in those fractions containing guanosine nucleotides. In all nucleotides the radioactivity was contained in the β- and γ-phosphorus atoms, since all radioactivity was liberated as inorganic phosphate upon hydrolysis for 10 min. at 100° in N-H_2SO_4. The activity of the individual fractions listed in Table 5 was not estimated under these conditions, for the quantities of nucleotides obtained from 100 g. of brain after processing as

Table 5. *Analysis of nucleotides of fraction* B *obtained from ox brain by separation of ammoniacal extracts on Dowex-1 chloride, and on charcoal followed by paper chromatography and electrophoresis*

For details see Methods section and text. Values are given as μmole/ml. of sample used.

Details of separation	Fraction	Identity of base	Base	Total P	Labile P	Pentose	Proposed identity
Separated in propanol–aq. NH_3 soln.–water (60:30:30, by vol.)	$C_{2(1)}$	Guanine	0·68	1·20	0·71	0·68	Guanosine diphosphate (GDP)
	$C_{2(2)}$	Guanine and adenine	0·45	1·10	0·71	0·42	Mixture of guanosine triphosphate (GTP) and adenosine diphosphate (ADP)
	$C_{3(1)}$	Guanine	0·26	0·55	0·25	0·19	Guanosine di- and tri-phosphates
	$C_{3(2)}$	Adenine	0·88	1·55	0·93	0·68	Adenosine di- and tri-phosphates
Separated by electrophoresis at pH 3·5	$C_{2(1),a}$	Guanine	0·11	0·24	0·11	0·10	Guanosine diphosphate
	$C_{3(1),b}$	Guanine	0·10	0·30	0·17	0·09	Guanosine triphosphate
	$C_{3(2),a}$	Adenine	0·40	0·79	0·42	0·40	Adenosine diphosphate
	$C_{2(2),b}$	Adenine	0·32	0·85	0·50	0·28	Adenosine triphosphate (ATP)

Table 6. *Effect of electrical pulses on the distribution of radioactivity in nucleotides isolated from guinea-pig cerebral slices and ox brain*

Nucleotide fractions were isolated from ox brain to which marker fractions, prepared from slices of guinea-pig cerebral cortex, had been added. For details see Methods section and text. Values are given as radioactivity of the phosphates (counts/min./μg. of P). Pulses, 18v peak potential, 0·4 msec. duration, were applied to slices furnishing the marker fractions for 10 sec.

Fraction	Identity*	Counts/min./μg. of P: With pulses	Counts/min./μg. of P: Without pulses
B	Mixed nucleotides	34·2	28·6
C_2	GDP, GTP, ADP	32·6	28·8
C_3	GTP, GDP, ADP, ATP	35·5	30·0
$C_{2(1)}$	GDP	20·1	16·0
$C_{2(2)}$	ADP, GTP	54·0	42·0
$C_{3(1)}$	GDP, GTP	40·0	26·8
$C_{3(2)}$	ADP, ATP	45·0	46·0

* See Table 5.

described above was small, and it became difficult to accumulate marker fractions of sufficient activity to be of use with larger quantities of brain. Also the length of time involved in working up the material further reduced the activity of the fractions.

DISCUSSION

Experiments detailed above were directed towards the isolation, from cerebral slices metabolizing radioactive inorganic phosphate, of phosphates which increased in specific radioactivity upon brief passage of electrical pulses through the slices. In this respect the experiments, while yielding a positive result in the finding of guanosine triphosphate as such a component, nevertheless yielded mainly negative results with respect to further characterization of the 'phosphoprotein' fraction originally sought.

The presence of guanosine di- and tri-phosphate in brain was first indicated by Kerr (1942), but no further evidence was obtained until Schmitz, Hurlbert & Potter (1954) briefly mentioned its presence in extracts of rat brain. More recently Thomas (1957) has found that nucleotides from extracts of guinea-pig cerebral slices yield guanine amounting to 0·3 μmole/g. wet wt. upon acid degradation. The identification of guanosine di- and tri-phosphate as cerebral nucleotides, described here, confirms Kerr's earlier deduction. The quantities of guanosine derivatives, 0·2–0·3 μmole/g. wet wt., found in the ammoniacal extracts constitute the total amount found by Thomas (1957) in guinea-pig brain, suggesting that guanosine di- and tri-phosphate are not extracted by the methods used above. This conclusion is rendered more

likely by failure to find guanosine in hydrolysates of the nucleotides precipitated from the ammonium sulphate extracts by the method of Heald & Long (1955). The quantity of guanosine derivatives relative to the total nucleotides present is much larger in brain than in muscle. Thus whereas in rat muscle guanosine triphosphate constitutes only 2–3% of the total nucleotides present (Bergkvist & Deutsch, 1953; Threlfall, 1957), in brain guanosine phosphates account for 18–20% of the total nucleotides both *in vivo* and *in vitro*. It seems reasonable to assume that in nervous tissue guanosine derivatives take part in some function more specific to the tissue than to non-nervous tissue. As isolated from ox brain the nucleotide fraction contained 0·26 μmole of adenine nucleotides/g. wet wt. or approx. 20% of the total normally present in cerebral tissues *in vitro* (Heald, 1956*a*). Whether these adenine nucleotides are present as contaminants due to difficulty in washing large quantities of cerebral extracts with saturated ammonium sulphate, or whether they constitute nucleotides metabolically distinct from those extracted by the ammonium sulphate, is not known. Experimental arrangements other than those described above would be needed to investigate this more fully.

The role of guanine nucleotides in a phosphate-transfer reaction of the type previously envisaged (Heald, 1956*b*) cannot be decided from the present evidence. Recently (Ayengar, Gibson, Peng & Sanadi, 1956), it has been shown that guanine nucleotides are involved in the phosphorylation of adenosine diphosphate to adenosine triphosphate during the breakdown of succinyl-coenzyme A. If such transphosphorylation occurs in cerebral tissue it must be the reverse of that described by Ayengar *et al.* for the specific radioactivity of guanine nucleotides increased on passing electrical pulses, whereas that of the adenine nucleotides decreases under similar conditions (Heald, 1956*b*).

Failure to obtain clear evidence of the presence of a phosphoprotein fraction also involved in the phosphate-exchange reaction can probably be attributed to the different conditions used. Previous demonstrations of the participation of phosphoprotein in the exchange (Heald, 1957*b*) were carried out upon tissue degraded in trichloroacetic acid, whereas the present experiments were carried out under alkaline conditions. It is possible that the phosphoprotein, demonstrated in tissue degraded under acid conditions, is in fact a stable degradation product of a more labile phosphate which is split to other products under alkaline conditions. Such a possibility has been considered by others (see, for example, Perlman, 1955) and it is of interest to note that, in so far as the determinations permitted, the inorganic phosphate found in the ammoniacal extracts of cerebral slices (Table 3) increased in specific radioactivity upon passage of pulses.

SUMMARY

1. Following upon a previous study, attempts have been made to isolate, from guinea-pig cerebral slices metabolizing radioactive inorganic phosphate *in vitro*, phosphates which increase in specific radioactivity upon the passage of electrical pulses through the slices.

2. A fraction possessing this property has been shown to consist of adenosine and guanosine di- and tri-phosphates, which were isolated and characterized. Only the guanine nucleotides increased in specific radioactivity upon the passage of pulses.

3. No other fraction was found which showed such an increase. It is suggested that the cerebral phosphoprotein fraction previously shown to increase in specific radioactivity under similar conditions is a stable degradation product of a more labile compound which is split to other products, possibly including inorganic phosphate, under the alkaline conditions used in the present experiment.

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The Use of Radioactive Isotopes in Immunological Investigations

12. THE BINDING OF A HAPTEN BY ANTIBODIES *IN VIVO**

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It is well known that haptens combine with antibodies *in vitro* (Marrack & Smith, 1932; Haurowitz & Breinl, 1933), although the forces which bind them together are comparatively weak. The *in vivo* neutralization of the physiological or pharmacological effects of haptens by antisera to antigens containing the corresponding determinant groups was demonstrated by Clutton, Harington & Yuill (1938) and by Butler, Harington & Yuill (1940). The former workers injected antisera to thyroxyl-proteins into rats and showed that in these rats thyroxine caused a smaller rise in the basal metabolic rate than in untreated rats. Butler *et al.* (1940) partially neutralized the antipyretic action of aspirin in rats by the prior injection of antisera to proteins that had been coupled with the azide of aspirin.

In the present study the binding of a hapten by antibodies *in vivo* has been inferred from experiments of a different type, in which the phenomenon has been investigated more directly. A radioactive hapten (3:5-di-iodotyrosine) has been injected into rabbits immunized against highly iodinated proteins [containing the 3:5-di-iodotyrosyl group as the main determinant group (Wormall, 1930)]. This injected hapten has been shown to persist longer in the plasma of these immunized rabbits than in the plasma of normal rabbits. Previous experiments along these lines by Mulligan (1950) were probably unsuccessful because large doses of di-iodotyrosine were injected, so that this compound was present in the plasma in considerable excess over the antibody.

MATERIALS AND METHODS

Isotopic labelling of di-iodotyrosine. The exchange reaction between di-iodotyrosine and I^- ion described by Miller, Anderson, Madison & Salley (1944) has been used to prepare small quantities of di-iodotyrosine of high specific activity. Di-iodotyrosine (1·2 mg., containing 5·06 μequiv. of I) was suspended in 1·0 ml. of 0·2M-sodium acetate–acetic acid buffer, pH 4·6, and the required quantity of carrier-free $Na^{131}I$ and 0·1 ml. of 0·01 M-KI (i.e. 1 μequiv.) added. This mixture, in a flask fitted with an air condenser, was then heated for 1 hr. on a boiling-water bath. After the mixture had been cooled, iodine was liberated from the inorganic iodide present by acidification with one drop of 10 N-H_2SO_4 and addition of 0·1 ml. of 0·01 N-KIO_3. The iodine was extracted with several 1 ml. portions of CCl_4, which were discarded, and the aqueous phase was then boiled briefly to free it of excess of CCl_4, cooled and neutralized to pH 7·4. Under these conditions about 80% of the added radioactivity has been found to appear in the di-iodotyrosine on several occasions when this preparation has been carried out.

The $Na^{131}I$ supplied by the Radiochemical Centre, Amersham, is dispensed in 0·02 M-$Na_2S_2O_3$ and it is essential that as little $S_2O_3^{2-}$ ions as possible should be introduced into the reaction mixture, as this substance inhibits the exchange reaction (cf. Gleason, 1955). In practice it has been found that as much as 0·2 ml. of 0·02 M-$Na_2S_2O_3$ (containing the $Na^{131}I$) can be used without lowering the recovery of ^{131}I in the di-iodotyrosine.

Since di-iodotyrosine leaves the blood stream very quickly after intravenous injection (Tong, Taurog & Chaikoff, 1954), it was necessary to use large amounts of ^{131}I in order to trace the hapten satisfactorily. Thus some rabbits received as much as 500 μC of ^{131}I, a dose which might be expected to have harmful biological effects if retained in the body for any length of time. However, Tong *et al.* (1954) have shown that 80% of an intravenously injected dose of di-iodotyrosine is excreted by rats in 24 hr., so that the injected rabbits were not likely to have suffered any harmful effect from the radiation.

Radioactivity measurements. These were made in scintillation counters of the type described by Banks, Tupper, Watts & Wormall (1955) coupled to a scaler and E.H.T. unit (Panax Ltd., Type 100C).

The radioactivity of all samples measured was such that they all gave net rates of counting which were above the background count rate. All counting was continued long enough to give a statistical accuracy of 2% or better.

Collection and fractionation of plasma. The solution of hapten was injected into the marginal vein of the right ear. Blood was collected from the marginal vein of the left ear into oxalated tubes and the plasma separated by centrifuging. Carrier di-iodotyrosine and KI were added to measured volumes of plasma and the proteins were precipitated by the addition of either 25 or 100% (w/v) trichloroacetic acid, so that the final trichloroacetic acid concentration was 10% (w/v). The precipitated proteins were centrifuged, washed thrice with 5 ml. of 10% trichloroacetic acid, dissolved in 1–2 ml. of N-NaOH and transferred to dishes for radioactivity measurements. The combined trichloroacetic acid washings were then treated with an amount of 0·02 M-KIO_3 sufficient to react with all the KI present, the liberated iodine was extracted into CCl_4

* Part 11: Francis & Hawkins (1957).

(3 × 2 ml.), and the radioactivity of the aqueous phase (containing di-iodotyrosine and related compounds) was measured. The iodine was extracted from the CCl_4 into 1 ml. of an appropriate concentration of $Na_2S_2O_3$, the CCl_4 was washed twice with a little water and these aqueous solutions were combined in dishes for radioactivity determinations. The efficiency of this procedure was demonstrated by preliminary experiments in which known amounts of radioactive di-iodotyrosine and $Na^{131}I$ were added to serum and recovered in the appropriate fraction. Table 1 gives the details of these experiments.

Immunization of animals. Rabbits were immunized with alum-precipitated highly iodinated bovine plasma albumin according to the method of Proom (1943) and used about 14 days after their last immunizing injection. Their sera were calibrated as described by Francis, Mulligan & Wormall (1954), the homologous antigen being used.

Calculation of results. The radioactivity (counts/min.) in each fraction of plasma was expressed as a percentage of the total radioactivity injected, assuming immediate and uniform distribution throughout the circulating plasma. Plasma volumes were determined by the use of Evans Blue, as described by Francis, Hawkins & Wormall (1957). In most cases the recovery of radioactivity in the various plasma fractions was checked by radioactivity measurements on the whole unfractionated plasma. The recoveries were usually satisfactory, the majority ranging from 90 to 105%. Any results outside these limits were discarded.

Table 1. *Recovery of* ^{131}I*-labelled di-iodotyrosine and* $^{131}I^-$ *from serum added*

	Radioactivity of compound added (counts/min.)	Radioactivity removed (counts/min.)	Recovery (%)
Di-iodotyrosine	1272	1239	97
Na I	2472	2351	95
Na I*	7476	7374	99

* Added to an antiserum to highly iodinated bovine albumin.

RESULTS

In some early experiments appreciable amounts of radioactivity were found in the protein fraction of the plasma from injected non-immunized rabbits. This must have arisen via the thyroid from the breakdown of the injected di-iodotyrosine. No comparable radioactivity was found associated with the plasma proteins of the immune animals, presumably because their thyroids were saturated with iodine derived from the breakdown of the antigen injected for immunization. In order to overcome this difference in behaviour between the normal and immune animals they were all given 0·05% of potassium iodide in their drinking water during the experiment and for the 5 days preceding it. This seemed to saturate the thyroids as judged by the fact that there was very little protein-bound ^{131}I in the plasma either of normal or of immune rabbits after the hapten injection.

Only small amounts of di-iodotyrosine were injected, so that it should not be present in excessive amounts relative to the circulating antibody. Thus any hapten bound to the antibody would constitute a significant percentage of the total present. The molar ratio of di-iodotyrosine to antibody aimed at was about 0·5. Thus the actual amounts injected into different rabbits varied according to the antibody content of their sera, but

Table 2. *Distribution of* ^{131}I *in rabbit plasma after the intravenous injection of* ^{131}I*-labelled di-iodotyrosine into three normal rabbits and three rabbits immunized against highly iodinated bovine plasma albumin*

All figures are expressed as the amount of ^{131}I present in the whole of the blood plasma, all the results being calculated as percentage of the amount of ^{131}I injected. Mean figures are given, together with the ranges. The total radioactivity injected varied slightly from animal to animal, but averaged $1·7 \times 10^7$ counts/min.

Time after injection (hr.)	Inorganic iodide		Protein-bound iodine		Residue*	
	Normal	Immune	Normal	Immune	Normal	Immune
3	4·4 (2·1–6·1)	7·8 (6·8–9·5)	0·39 (0·24–0·58)	0·54 (0·45–0·60)	1·6 (0·8–2·6)	1·8 (1·3–2·0)
6	4·0 (1·3–5·7)	7·0 (5·5–8·5)	0·15 (0·11–0·18)	0·27 (0·21–0·31)	0·43 (0·18–0·72)	0·79 (0·63–0·91)
9	2·6 (0·8–3·7)	5·9 (4·5–7·3)	0·17 (0·10–0·22)	0·27 (0·26–0·30)	0·23 (0·10–0·41)	0·42 (0·30–0·49)
11	2·1 (0·6–3·1)	4·7 (3·3–5·5)	0·13 (0·08–0·19)	0·22 (0·18–0·24)	0·27 (0·12–0·47)	0·44 (0·44–0·44)
24	1·2 (0·3–2·8)	3·3 (1·9–4·9)	0·07 (0·06–0·07)	0·09 (0·07–0·10)	0·10 (0·05–0·21)	0·14 (0·09–0·19)
32	1·1 (0·1–2·8)	2·5 (1·3–3·8)	0·05 (0·04–0·07)	0·09 (0·07–0·10)	0·06 (0·001–0·15)	0·11 (0·05–0·19)
48	1·0 (0·02–2·6)	0·9 (0·2–1·7)	0·03 (0·02–0·04)	0·04 (0·03–0·05)	0·04 (0·002–0·01)	0·09 (0·03–0·16)

* Assumed to be mainly di-iodotyrosine (see text).

the usual injection level was about 100 μg. of di-iodotyrosine/kg. body weight. Control rabbits were also injected with slightly different amounts of the hapten so as to correspond as closely as possible with the different immune rabbits. However, no evidence was found of differences in behaviour due to the injection of different amounts of di-iodotyrosine within the range studied.

The results of a typical experiment with three immunized and three non-immunized rabbits are recorded in Table 2. The results show a wide scatter. However, very similar results were observed in two other experiments (in one of which the persistence of the hapten was observed in whole blood rather than in plasma), so it seems highly probable that the differences between the two groups of animals are real. Plasma samples from the immune animals in the first 24 hr. after injection of the di-iodotyrosine contained more di-iodotyrosine than the corresponding samples from the normal rabbits. Thereafter there was no significant difference between the two groups, but by that time the concentration of di-iodotyrosine in the plasma was extremely low. An unexpected observation was the presence of larger amounts of radioactive inorganic iodide in the plasma of the immune animals than in that of the normal ones. This must have arisen from the breakdown of the injected di-iodotyrosine and suggests that this substance is catabolized faster in the immune animals than in the normal ones.

DISCUSSION

Tong *et al.* (1954) have shown that ^{131}I-labelled di-iodotyrosine is very rapidly broken down after intravenous injection into rats, so that 1½ hr. after injection nearly all the ^{131}I in the blood is in the form of inorganic iodide. Thus after injection of di-iodotyrosine it is necessary to fractionate the blood or plasma in order to determine how much di-iodotyrosine is actually present. The designations protein-bound iodine and inorganic iodide applied here to two of the fractions are clear-cut and reliable. The designation of the residue after removal of these two fractions as di-iodotyrosine is less certain, for Tong *et al.* (1954) have shown that compounds such as 4-hydroxy-3:5-di-iodophenyllactic acid and 4-hydroxy-3:5-di-iodophenylpyruvic acid may be found in rat's plasma after the injection of di-iodotyrosine. However, these compounds both contain the 4-hydroxy-3:5-di-iodophenyl group which is the determinant group of di-iodotyrosine and would therefore be expected to possess similar, if not identical, haptenic properties (cf. Snapper & Grunbaum, 1936). It therefore seems reasonable to conclude that the residual fraction, even if not an accurate measure of di-iodotyrosine, is a measure of substances that will function as haptens in the serological system under consideration.

The demonstration that more hapten is circulating in the plasma of the immune animals than of the normal animals during the first 24 hr. after injection provides definite, if somewhat indirect, evidence for the combination of hapten and antibody *in vivo*. The amount of hapten combined in this way seems to be extremely small under the conditions reported here. Thus in the experiment described in Table 2, the actual amounts of di-iodotyrosine in the plasma of the immune rabbits 6 hr. after the injection varied between 0·019 and 0·027 μm-mole/ml., whereas in the normal animals the corresponding figures were 0·008 and 0·022 (mean 0·014) μm-mole/ml. Thus the actual amount bound to antibody is likely to have been of the order of only 0·01 μm-mole/ml. The antibody concentration in the rabbits' serum varied between 7 and 15 μm-moles/ml. and was about a thousand times as great. When larger amounts of di-iodotyrosine (about 1 mg./kg. body weight; molar ratio of di-iodotyrosine to antibody, approx. 4) were injected into another group of rabbits, the absolute differences between the amounts in the plasmas of normal and immune rabbits were greater, and about 0·05 μm-mole was bound to the antibody in 1 ml. of plasma. It seems probable that if still larger amounts of di-iodotyrosine were injected greater amounts would be bound to antibody, in correspondence with the known behaviour of *in vitro* systems. Owing to the comparatively low solubility of this compound at pH 7, this would not be possible without injecting very large volumes of solution.

The presence of more inorganic iodide in the plasma of the immune rabbits than in that of the normal ones is unexpected and its significance is not certain. It seems unlikely that there would be any difference between the two groups of animals in the rate of excretion of this substance, and if some of the di-iodotyrosine is bound to the antibody in the plasma it might be expected that it would be protected to a certain extent from catabolism in the immune rabbits. A possible explanation is that the immune rabbits might have become adapted in some way to catabolize di-iodotyrosine more rapidly than the normal rabbits. This would be a most interesting manifestation of the immune response, and this possibility is worthy of further investigation.

SUMMARY

1. The persistence in the blood plasma of various fractions containing ^{131}I has been determined after the intravenous injection of ^{131}I-labelled di-iodotyrosine into normal rabbits and rabbits immunized against highly iodinated bovine plasma albumin.

2. During the first 24 hr. after injection a substance whose properties resemble those of

di-iodotyrosine is present in higher concentration in the plasma of the immunized rabbits than in that of the normal ones. It is suggested that this is due to combination of the hapten with antibodies in the plasma of the immune animals.

3. During the same period inorganic iodide is also present in higher concentration in the plasma of the immunized rabbits than in that of the normal ones.

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The Mechanism of Carbohydrase Action

3. THE ACTION PATTERN OF β-AMYLASE*

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β-Amylase is a plant enzyme which hydrolyses α-1:4-linked polymers of D-glucopyranose by an endwise action. This action commences at the non-reducing end of the polymer, and the penultimate and subsequent alternate linkages are broken to yield maltose. These facts being established, there are two possible mechanisms for the enzyme action. These are a single-chain mechanism in which the enzyme completely hydrolyses one substrate molecule at a time or a multi-chain mechanism in which the enzyme attacks the ends of the polymer chain in a completely random fashion. *A priori* considerations suggest that single-chain action is unlikely, but the first investigations of the problem provided evidence in its favour (Swanson, 1948; Cleveland & Kerr, 1948). These authors' conclusions were criticized by Hopkins & Jelinek (1949) and by Bourne & Whelan (1950), both groups of authors favouring multi-chain action. More recently, Kerr & Cleveland (1951) have provided further arguments in favour of single-chain action, while Hopkins & Jelinek (1954) and Bird & Hopkins (1954) take the contrary view.

There have been two methods of approach to the problem. The first is that used by Swanson (1948), Cleveland & Kerr (1948) and Kerr & Cleveland (1951). This employs native amylose as the enzyme substrate. The polysaccharide remaining at stages in the hydrolysis is isolated and its properties are compared with those of the original material. If the action is single-chain in character this residual polysaccharide should be unchanged amylose. In the second method, instituted by French, Levine, Pazur & Norberg (1950*b*), a much smaller substrate molecule is used in order to facilitate tests for the production of intermediate products. Using maltoheptaose, French *et al.* (1950*b*) observed that under optimum conditions for the enzyme action maltoheptaose was degraded to maltose and maltotriose without the intermediary formation of maltopentaose, i.e. the action was apparently single-chain. At

* Part 2: Whelan & Roberts (1953).

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extremes of temperature and pH, however, maltopentaose formation was detected (French, Knapp & Pazur, 1950*a*). These results were interpreted to mean that 'either the enzyme–substrate affinity constants are seriously decreased at higher temperatures or unfavourable pH, or the rate at which the enzyme–substrate complex disintegrates into products is diminished, or possibly a combination of these two effects'. In the present investigation of β-amylase action we have used both short-chain dextrins (maltoheptaose and maltohexaose) and also a synthetic amylose of average chain length 49 units as substrates for the enzyme.

EXPERIMENTAL

Materials

Enzymes. Soya-bean β-amylase was prepared as by Peat, Pirt & Whelan (1952*b*). Crystalline sweet-potato β-amylase was the gift of Dr A. K. Balls. Potato phosphorylase was prepared as by Whelan & Bailey (1954).

Maltodextrins. The preparation of these substances from potato amylose is described by Whelan, Bailey & Roberts (1953).

Synthetic amylose. This was prepared from maltohexaose primer as by Whelan & Bailey (1954) and had an average degree of polymerization (DP) of 49 glucose units. Concentrations of the amylose solutions were determined by acid hydrolysis to glucose (Pirt & Whelan, 1951).

Buffers. The components of the four buffer systems used were sodium acetate–acetic acid, NaH_2PO_4–sodium citrate (McIlvaine, 1921), NaH_2PO_4–Na_2HPO_4 and HBO_3–$Na_2B_4O_7$.

Methods

All concentrations of digest components given were those finally obtaining in the digests.

The method of Somogyi (1945) was used for determining reducing powers. β-Amylase activity was determined and units defined as by Hobson, Whelan & Peat (1950). The solution used for iodine staining contained 0·2% I_2 in 2% (w/v) KI. Light-absorption of stained solutions was measured in 1 cm. cells in a Unicam SP. 500 spectrophotometer.

Relative rates of β-amylolysis of starch and maltodextrins. Each digest (5·5 ml.) contained malto-tetraose, -pentaose, -hexaose or -heptaose (3·7 μmoles) or potato starch (14·9 mg.), 17 mM-acetate buffer (pH 4·8) and crystalline β-amylase (0·1 unit). Measurements of reducing power (1·5 ml. portions) were made after incubation at 35° for 5, 15 and 30 min. and the initial reducing power of each substrate was also determined. The reducing power/time curves were plotted and tangents drawn to the curves to determine the approximate initial relative rates of hydrolysis. Relative to potato starch (=100), these were maltotetraose, 30; -pentaose, 30; -hexaose, 75; -heptaose, 85.

Small-scale fractionation of maltodextrin mixtures. A mixture of maltose hydrate (11·0 mg.), malto-triose (5·5 mg.), -pentaose (21·0 mg.) and -heptaose (14·5 mg.) was placed on a charcoal–Celite column (38 cm. × 1·5 cm.). The sugars were separated by successive elution with 7·5, 15, 22 and 28% (w/v) aqueous ethanol, fractions of 20 ml. being collected. The optical rotation of each fraction was measured in a 4 dm. tube and the recovery of each sugar estimated in terms of its specific optical rotation (Whelan *et al.* 1953). The percentage recoveries were: maltose, 100; malto-triose, 87; -pentaose, 75; -heptaose, 70. The overall recovery on a weight basis was 81%.

Determination of extent of multi-chain action on maltodextrins. The following procedure was adopted in each experiment. A small-scale digest was used to determine the time necessary to attain the desired conversion, the range 50–70% being suitable for obtaining the maximum yield of intermediate. A larger-scale digestion was then carried out for the required time and the enzyme was rapidly inactivated by immersing the digest in a boiling-water bath. When the digestion was at 0·5°, $HgCl_2$ was added as an additional means of securing rapid inactivation.

The two maltoheptaose digestions (Table 1) were carried out at 35° as follows. The digests (50 ml. each) contained maltoheptaose (32 mg.), 20 mM-acetate buffer (pH 4·8) or McIlvaine buffer (pH 8·03, in fivefold dilution) and crystalline β-amylase (1 and 8 units respectively). The times of incubation were 32 and 10 min. respectively. After the enzyme had been inactivated, the reducing powers were measured on 2 ml. portions and the rest of the digests fractionated on charcoal–Celite as above. The respective overall recoveries of sugar were 81 and 87% (see Table 2).

The three maltohexaose digests contained substrate (for wt. see Table 2) and purified soya-bean β-amylase in 40 mM-acetate buffer (49 ml.). One digest (200 units of enzyme) was incubated at 0·5° for 19 min., when $HgCl_2$ (20 mg., 1 ml.) was added to inactivate the enzyme, followed by heating to 100°. The second digest (20 units of enzyme) was incubated for 20 min. at 35° followed by heat inactivation. The third (120 units of enzyme) was kept at 70° for 10 min. before the temperature was raised to 100°. A portion (1 ml.) was removed from each digest to determine the extent of conversion (Table 2) and the remainder was fractionated on charcoal as previously described. The overall recoveries of carbohydrate for the different experiments were 0·5°, 89%; 35°, 76%; 70°, 91%.

Calculation of proportion of multi-chain action during β-amylolysis of malto-heptaose and -hexaose. The following mathematical treatment requires that the reactions under consideration should obey first-order reaction kinetics. This was so for each of the substrates under the conditions employed.

The multi-chain action of β-amylase on maltoheptaose corresponds to a two-stage consecutive reaction:

$$\mathrm{M{-}M{-}T} \xrightarrow{k_1} \mathrm{M + M{-}T} \xrightarrow{k_2} \mathrm{2M + T},$$

where M = maltose, T = maltotriose and k_1, k_2 are the respective rate constants for the β-amylolysis of maltoheptaose and -pentaose. If the initial amount of maltoheptaose is given by a, then the amount of unchanged maltoheptaose at time t is given by $ae^{-k_1 t}$ (denoted as x) and of maltopentaose by $ak_1(e^{-k_1 t} - e^{-k_2 t})/(k_2 - k_1)$ (denoted subsequently as y) (Daniels, 1948). At complete conversion of maltoheptaose 2 mol. prop. of maltose has been liberated. Therefore the measured increase in reducing power taking place during complete hydrolysis is proportional to $2a$. On the same basis the increase in reducing power at any intermediate stage is proportional to $2(a-x)-y$. The measured fractional conversion into maltose is therefore

equal to $[2(a-x)-y]/2a$. By using the known values of a (Table 2) and k_1/k_2 (see above), this last equation was solved graphically for t in terms of k_1 or k_2. The value of t was then substituted in the expression for y (the k terms cancel) giving the amount of maltopentaose at time t, and hence at the measured degree of conversion. This is the amount theoretically obtainable on the basis of completely multi-chain action. It was compared with the actual amounts found by direct analysis of the digest mixture as already described. It is also possible to calculate the theoretical amount of unchanged maltoheptaose from x; of maltose formed, from $2(a-x)-y$; and of maltotriose formed, from $a-x-y$. Similar calculations were applied in the experiments where maltohexaose was used.

Phosphorolytic degradation of synthetic amylose. Each digest (16 ml.) contained 0·06% of synthetic amylose, 63 mM-phosphate buffer (pH 7·0), 0·47% of freeze-dried potato phosphorylase, μM-$HgCl_2$ and 0·45% of ammonium molybdate [see Bailey, Thomas & Whelan (1951) concerning the use of $HgCl_2$ and molybdate as enzyme inhibitors]. The digests were incubated at 0°, 35° and 55° respectively, and at zero time and subsequent intervals, according to the rate of phosphorolysis, portions (1 ml.) of the digests were withdrawn and stained with iodine (0·5 ml.) in a total volume of 25 ml. The light-absorption in the range 500–640 mμ was determined for each iodine-stained solution. Figs. 1 and 2 show the patterns of light-absorption curves obtained during the synthesis of this polysaccharide and during phosphorolysis at 35°. The slope of the line joining the peaks of each family of curves was measured. The slope for each temperature is plotted in Fig. 5.

β-Amylolysis of synthetic amylose. Four types of experiment were performed.

(i) The digests contained 0·08% of amylose in 24 mM-acetate buffer (pH 4·8, 12·5 ml.). After equilibration at the desired temperature a portion (0·85 ml.) was stained with iodine (0·5 ml.) in 25 ml. and to the remainder of the digest was added purified soya-bean β-amylase. Portions (0·85 ml.) were subsequently removed for iodine staining. No correction was applied for the slight dilution of the digest caused by the addition of the enzyme. The light-absorption of each solution in the range 500–800 mμ was determined. Table 1 summarizes the details of the experiments and the results obtained are shown in Figs. 3 and 5.

(ii) A digestion was carried out as in (i) except that borate buffer (pH 8·1) replaced acetate. The digest contained 180 units (0·2 ml.) of enzyme. Eight observations were made during 43 min. (see Fig. 5).

(iii) In order to examine the reversibility of heat effects on the enzyme, β-amylase solution (0·5 ml.; pH 4·8; 450 units) was heated at 70° for 5 min., cooled rapidly to 35° and 0·2 ml. of the solution used in an otherwise exact repetition of the first type of experiment at 35°. Six observations were made during 6 min. incubation (see Fig. 5).

(iv) The action of the enzyme at 35° was then examined in the presence of a 130-fold molar excess (over synthetic amylose) of maltotetraose. The only other difference in conditions in this latter experiment was that 6 units (0·1 ml.) of enzyme were added initially, followed by a further 30 units (0·1 ml.) after 4·2 min. Seven observations were made during 8·25 min. (see Fig. 5).

Optimum temperature of action of soya-bean β-amylase. Each of five digests (50 ml.) contained 0·6% soluble potato starch, 24 mM-acetate buffer (pH 4·8) and purified soya-bean β-amylase (30 units). The digests were brought to the incubation temperature before adding and mixing the enzyme (1 ml.). Portions (5 ml. each) were removed after 3 and 8 min., the enzyme action being arrested by mixing immediately with Somogyi (1945) copper reagent (5 ml.) for determination of reducing power (as for maltose) which values are expressed in Fig. 6 in terms of the 5 mM-thiosulphate used in the Somogyi method.

Examination of the dialysable products of β-amylolysis. The detailed results of these experiments are not given since they were negative. The methods were as follows. Potato amylopectin was incubated with crystalline β-amylase at 35° and 70°, both at pH 4·8. At six intervals, during the time taken to attain limiting β-amylolysis of the amylopectin, inactivated portions of the digests were dialysed into water and the diffusible material was examined on paper chromatograms. No product other than maltose was detected.

RESULTS AND DISCUSSION

Outline of methods

We have used two types of low-molecular-weight substrate in this investigation. These are: (i) pure maltodextrins produced from amylose by acidic hydrolysis (Whelan *et al.* 1953); and (ii) synthetic amylose of known homogeneity and average degree of polymerization (DP), prepared from maltohexaose and glucose 1-phosphate by phosphorylase action. The preparation of such polymers of known properties has become possible as a result of the proof that potato phosphorylase functions by multi-chain action (Whelan & Bailey, 1954).

Table 1. *Experimental conditions in the β-amylolysis of synthetic amylose*

The measurements were made at irregular intervals during the stated period (see e.g. Fig. 3).

	Temp. of incubation							
	0°	5°	21°*	21°*	35°	55°	70°†	75°‡
β-Amylase solution (ml.)	0·1	0·2	0·1	0·1	0·1	0·1	0·012	0·024
Units of activity	90	180	9	45	9	14	6	4
Total no. of observations	12	4	11	7	10	6	12	7
Time of last observation (min.)	27·5	6·0	43	8·75	22·5	5·0	22·5	18·2

* Low and high enzyme: substrate ratios (Fig. 5).
† Because of heat inactivation a further portion of enzyme (0·012 ml.; 3 units) was added after 15 min.
‡ Because of heat inactivation the same amount of enzyme was added a further six times during incubation.

By using these two types of substrate it is possible to predict in advance the changes which would be observed during single-chain and multi-chain action when the course of reaction is followed by iodine staining (for synthetic amylose substrate) or by charcoal-chromatographic analysis (for malto-dextrin substrate). During single-chain action the substances present at any stage will be maltose and unchanged substrate. Intermediary products in course of degradation may be neglected since the magnitude of the turnover number of β-amylase (Englard & Singer, 1950; Englard, Sorof & Singer, 1951) is such that in any β-amylolysis proceeding at a measurably slow rate the molar ratio of enzyme to substrate is negligibly small. Therefore in the course of the single-chain degradation of malto-hexaose and -heptaose one would not expect to detect malto-tetraose or -pentaose respectively. During the single-chain β-amylolysis of a synthetic amylose the changes in iodine stain should correspond to those of increasingly dilute solutions of the original substrate, an important point being that the wavelength of maximum light-absorption ($\lambda_{max.}$) of the iodine complex should not change.

Whereas the patterns of single-chain action can be predicted, certain experimental measurements must be made to establish the characteristics of multi-chain action. For the achroic maltodextrins it is necessary to know the relative rates of β-amylolysis of the substrate and of substrate molecules containing 2, 4, etc. fewer glucose units. The simplest suitable substrates are malto-hexaose and -heptaose, from each of which only two molecules of maltose can be removed. Each can therefore give rise to only one multi-chain intermediate, namely malto-tetraose and -pentaose respectively. The relative rates of β-amylolysis of malto-tetraose, -pentaose, -hexaose and -heptaose were measured. The amount of intermediate theoretically obtainable by multi-chain action was then calculated for the particular degree of hydrolysis as shown in the Experimental section. The actual amounts of intermediate present in our experiments were determined by charcoal-chromatographic fractionation of the products of partial degradation. In this way the enzyme action under given conditions was characterized in terms of the proportion of intermediate actually detected, as compared with that calculated on the basis of completely multi-chain action (Table 2).

The characteristics of multi-chain degradation of the synthetic amylose (DP 49) were established by subjecting it to phosphorolytic degradation with the potato enzyme and measuring the light-absorption of the iodine-stained intermediate products. As is mentioned above, it has been shown that potato phosphorylase functions by an exclusively multi-chain action pattern. The iodine-staining method is only suitable for affording a clear differentiation between single- and multi-chain action if the DP of the substrate is less than about 80 glucose units, since molecules of greater size show little difference in iodine-staining properties (Bailey, J. M. & Whelan, W. J. unpubl. work). Consequently if natural amylose (DP at least several hundred) is used (as by Swanson, 1948; Cleveland & Kerr, 1948; Kerr & Cleveland, 1951) it is impossible, except in the final stages of the enzymic degradation, to determine by iodine staining whether all the chain molecules are being degraded (multi-chain action) or are disappearing one at a time (single-chain action) (Bourne & Whelan, 1950).

Two specimens of β-amylase were used. One was a purified preparation from soya bean (Peat *et al.* 1952*b*), the other was the crystalline sweet-potato enzyme (Balls, Walden & Thompson, 1948). There appear to be no differences in the enzymic properties of the two preparations (Peat *et al.* 1952*a*, *b*) and they have therefore been used interchangeably.

β-Amylolysis of maltodextrins

β-Amylase was allowed to degrade malto-heptaose or -hexaose to intermediate stages under varying conditions of temperature and pH. The products were separated and the amounts of malto-pentaose or -tetraose intermediate obtained were compared with the amounts calculated for completely multi-chain action. The results are summarized in Table 2.

Table 2. *Action of β-amylase on malto-heptaose and -hexaose*

See Experimental section for details.

Expt. no.	Source of enzyme	Substrate	Wt. of substrate (mg.)	Temp.	pH	Conversion into maltose (%)	Recovery of intermediate (mg.) (*A*)	Calc. recovery* (mg.) (*B*)	Proportion of multi-chain action (100*A*/*B*)
1	Sweet potato	Maltoheptaose	32·0	35°	4·8	52	3·3	12·6	26
2			32·0	35°	8·0	58	4·1	11·7	35
3	Soya bean	Maltohexaose	92·7	35°	4·8	64·5	25·3	45·7	56
4			97·1	70°	4·8	48	27·1	48·8	56
5			92·7	0·5°	4·8	69·5	35·6	36·4	98

* Calculated on the assumption of completely multi-chain action.

These results show that even under optimum conditions there is an appreciable proportion of multi-chain action (Expts. 1 and 3). This finding conflicts with the results of French *et al.* (1950*b*), mentioned above, but the explanation is probably in our use of a more selective fractionation technique. French *et al.* employed electrophoretic fractionation of the hypoiodite-oxidized products. In agreement with French *et al.* (1950*a*), an unfavourable pH increased the proportion of multi-chain action over that obtaining at optimum pH (Expts. 1 and 2). Raising the temperature above the optimum did not increase the multi-chain action (Expts. 3 and 4) but at 0·5° (Expt. 5) the action on maltohexaose was almost completely multi-chain.

The results show that under optimum conditions the enzyme action displays characteristics intermediate between the extreme types and they provide further insight into the enzyme mechanism by demonstrating the dependence of the action pattern on environmental conditions. While these results alone might form the basis of deductions concerning the mechanism of the enzyme action, it was thought desirable to provide confirmatory evidence with a larger molecule as substrate for the following reasons: (*a*) Limiting substrates probably lead to an unusually high accumulation of intermediate (e.g. the removal of the second molecule of maltose from malto-heptaose and -hexaose is accomplished two to three times as slowly as that of the first molecule); (*b*) an increase of one glucose unit in the size of the substrate was accompanied by a fall from 56 to 26 in the percentage of multi-chain action (Expts. 1 and 3). Such 'end' effects should be much less prominent in larger molecules.

β-Amylolysis of synthetic amylose

A synthetic amylose (DP 49) was used as substrate for β-amylase and for phosphorylase under varying conditions of temperature and pH. The progress of degradation was measured by iodine-staining, the absorption curves of the coloured complexes being plotted in the region 500–700 mμ. It was found that a straight line could be used to join the peaks (i.e. $\lambda_{max.}$ values) of the curves. Two values of wavelength were determined. These were $\lambda_{max.}$ at zero time, t_0, and the wavelength at which the projection of the straight line cut the wavelength axis, i.e. at zero extinction, E (e.g. see Fig. 2). Since the same polysaccharide was used in all the experiments the slopes of the various straight lines could be compared in terms of ($\lambda_{max.}$ at $t_0 - \lambda$ at $E = 0$). The value of the slope was used to characterize the type of degradation under any given set of conditions. Four such patterns of absorption curves are shown, one for phosphorylase synthesis (Fig. 1), one for phosphorolytic (Fig. 2) and one for β-amylolytic degradation (Fig. 3) of the 49-unit amylose. Fig. 4 shows the pattern of curves expected for single-chain degradation, and Fig. 5 summarizes the results of a number of experiments.

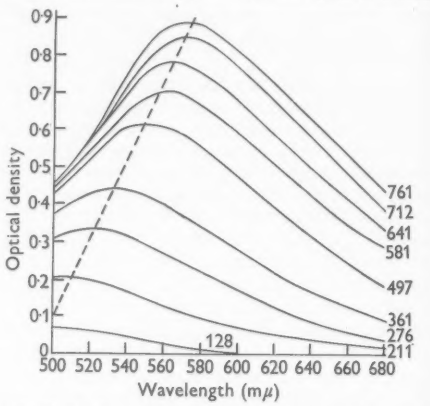


Fig. 1. Light-absorption curves of iodine-stained products during the phosphorylase-catalysed synthesis of amylose (DP 49) from maltohexaose primer and glucose 1-phosphate at 35°, pH 7·0. Equal volumes of the digest were stained during the reaction and the uppermost curve is that given by the final product when the reaction was terminated by heat inactivation of the enzyme. The numerals against the curves refer to the time of incubation (min.). The digest contained 0·18% freeze-dried potato phosphorylase. After 172 min. the concentration was increased to 0·22%. Experimental conditions are given by Whelan & Bailey (1954).

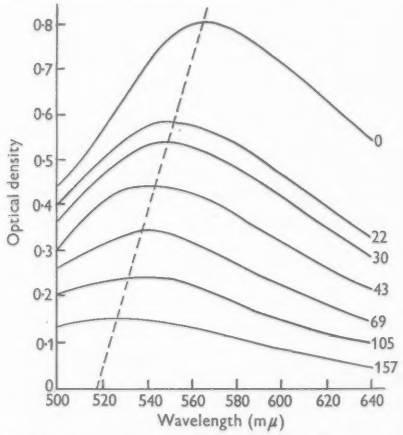


Fig. 2. Phosphorolysis of synthetic amylose (DP 49) at 35°, pH 7·0. Light-absorption curves of iodine-stained products. The numerals against the curves refer to the time of incubation (min.). Conditions are given in the Experimental section.

As explained above, single-chain degradation of the polysaccharide by β-amylase would give a pattern of curves with zero slope (see Fig. 4), whereas the slope for completely multi-chain degradation is given by the curves for phosphorolytic action (Fig. 2). The slope of the phosphorylase curves was found not to be subject to temperature variation (Fig. 5). In view of the comparative behaviour of β-amylase, discussed below, this is a strong indication that phosphorylase action is completely multi-chain at all temperatures (cf. Whelan & Bailey, 1954). The fact that the slope during phosphorolytic degradation is less than the slope during phosphorylase synthesis of the polysaccharide (Figs. 1 and 2) does not mean that there is any difference in mechanism between the two types of action. The reason for the change in slope has been discussed by us elsewhere (Whelan & Bailey, 1954).

As regards β-amylase, it is evident from Fig. 5 that under none of the conditions investigated does the enzyme act by completely multi-chain or completely single-chain action; the proportion of multi-chain action is greatest at extremes of temperature

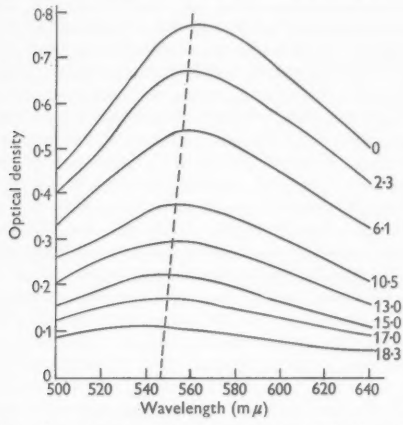


Fig. 3. β-Amylolysis of synthetic amylose (DP 49) at 35°, pH 4·8. Light-absorption curves of iodine-stained products. The numerals against the curves refer to the time of incubation (min.). Conditions are given in the Experimental section.

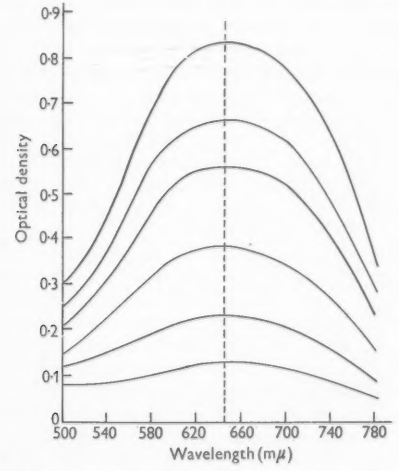


Fig. 4. Family of curves representing the changes in iodine-staining properties of amylose which should occur during β-amylolysis by single-chain action. The curves actually refer to potato-amylose solutions of increasing dilution.

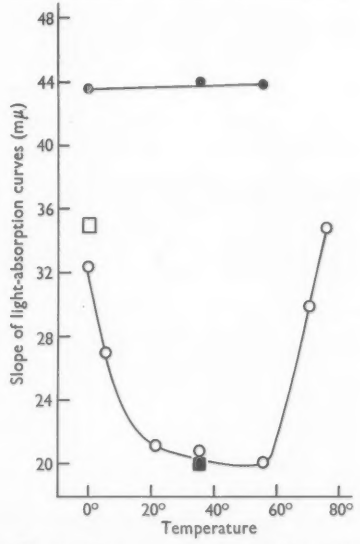
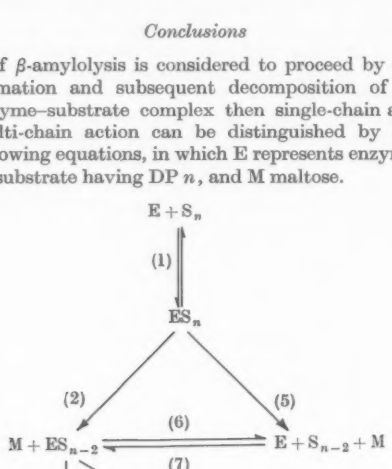


Fig. 5. Effects of temperature on the action patterns of soya-bean β-amylase and potato phosphorylase. 'Slope of light-absorption curves' refers to the slope of the line joining the maxima of the light-absorption curves of the iodine-stained products of β-amylolysis and phosphorolysis of synthetic amylose (DP 49) (see Figs. 2, 3). The method of calculating the slope is explained in the Results section. The value would be zero for completely single-chain action. ●, Phosphorolysis at pH 7·0; ○, β-amylolysis at pH 4·8; □, β-amylolysis at pH 8·1; ■, β-amylolysis in the presence of 130-fold molar excess of maltotetraose and β-amylolysis, with heat-treated enzyme, both at pH 4·8 (the two points are coincident). The experimental points at 21°, pH 4·8 for β-amylolysis at two different concentrations of enzyme are coincident. Details are given in the Experimental section.

or pH, or both, and least under optimum conditions. The overall behaviour of the enzyme is in agreement with the results already obtained with the two maltodextrins (Table 2), except that no difference in action pattern on maltohexaose was found between 35° and 70°. This is probably due to the difference in size of the substrates used in the two types of experiment (see above).

Conclusions

If β-amylolysis is considered to proceed by the formation and subsequent decomposition of an enzyme–substrate complex then single-chain and multi-chain action can be distinguished by the following equations, in which E represents enzyme, S_n substrate having DP n, and M maltose.



Single-chain action is the result of the successive reactions (1), (2), (3), (4) and so on, in which the same enzyme molecule reacts with the same substrate molecule. Multi-chain action proceeds either by reactions (1) and (5), or (1), (2) and (6), followed, in reaction (7), by recombination of the enzyme with the same or with a different substrate molecule. Our results show that under most conditions the action pattern is intermediate between single- and multi-chain. The question therefore arises whether the effects of reaction conditions in varying the proportion of the two types of action can be interpreted in terms of these equations.

One possibility which was eliminated was that the reaction is essentially multi-chain but that the rate of β-amylase action is so large that a substrate molecule which has just undergone attack is unable to diffuse away with sufficient rapidity to avoid a second encounter. To test this hypothesis we compared the action patterns at 35° of the enzyme reacting with synthetic amylose in the presence and absence of a 130-fold molar excess of maltotetraose. The added sugar had no effect on the proportion of multi-chain action (Fig. 5). On the grounds that maltotetraose must be capable of competing with synthetic amylose for the enzyme in any diffusion process, it was concluded that reactions (1), (2), etc., can occur successively without dissociation of the enzyme–amylose complex. β-Amylase action therefore involves combination of the enzyme with the substrate molecule followed by the removal of a number of maltose units (reactions 1, 2, 3, 4, etc.) before the enzyme–substrate complex decomposes. There are two ways in which this can happen; either the enzyme molecule contains several active centres, or multiple successive reactions can occur at the same active centre. No distinction between these possibilities can be made on the basis of the present data. It is clear, however, that with the substrates tested, the number of maltose units removed per encounter cannot be very large, for if this number were never less than two, no intermediate malto-tetraose or -pentaose would have been detected in the degradation of malto-hexaose and -heptaose, respectively.

The most reasonable explanation of the temperature-induced changes in the action pattern is that it involves changes in the average number of maltose units removed per encounter, brought about by changes in the *relative* values of the reaction constants governing reactions (1), (2), (3), (4), etc. The physical reasons for these relative changes in rate

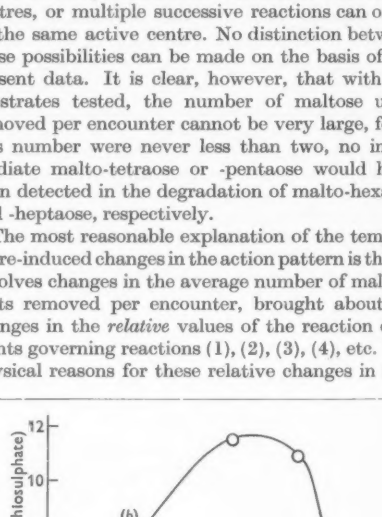


Fig. 6. Activity/temperature relationship for soya-bean β-amylase acting on potato starch at pH 4·8. Details are given in the Experimental section. The curves show the extent of action after (*a*) 3 min. and (*b*) 8 min.

constants are not clear but alterations in the configuration of the enzyme or substrate are possible lines for exploration. The variations in action patterns bear a strong resemblance to the variation in rate of action of the enzyme with temperature (Figs. 5, 6), but although the increases in rate of action of the enzyme and in proportion of single-chain action at the lower temperatures are probably due to related causes, it is not considered that there is any such connexion at the higher temperatures. Although the extent of enzyme action is decreased at high temperatures the activity of an individual enzyme molecule should continue to increase with temperature for as long as it retains its catalytic power. It is shown in Fig. 5 that a five-fold variation in the concentration of enzyme did not influence the action pattern and a test was made for the possibility that at high temperatures the enzyme passes through a less active stage in the process of denaturation. If such a change were irreversible then the less active form could be prepared by heat treatment. It was observed, however, that a specimen of enzyme which had been heated to 70° had the same type of action at 35° as did an unheated specimen (Fig. 5).

The investigation now reported has been extended by an alternative method of approach using synthetic amyloses labelled with ^{14}C at the non-reducing chain ends (Bailey & French, 1957). Results with crystalline sweet-potato β-amylase indicate that at 35°, pH 4·8, the enzyme removes, on average, four units of maltose per enzyme–substrate encounter.

SUMMARY

1. The action pattern of β-amylase has been studied under a variety of conditions using as substrates two maltodextrins and a synthetic amylose of average chain length 49 units.

2. With all the substrates and under all conditions the action pattern was always intermediate between single- and multi-chain. With maltohexaose the highest proportion of multi-chain action (98 %) was at 0·5°, pH 4·8; the proportion was much less at 35° and was the same at 70° as at 35°. With synthetic amylose at optimum pH the proportion of multi-chain action decreased continuously between 0° and 20°, remained approximately constant between 20° and 60° and increased between 60° and 75°. The proportion was also increased when the pH was changed from the optimum.

3. The action pattern on synthetic amylose was not significantly influenced by the addition of a 130-fold molar excess of maltotetraose.

4. The results show that the enzyme removes several maltose units during each encounter with a substrate molecule. The data using malto-hexaose and -heptaose indicate that this number is small. One explanation of the variation in action pattern induced by changes in temperature and pH is that it represents a change in the average number of maltose units removed per encounter. It is suggested either that the enzyme molecule contains several active centres or that multiple reactions can occur at a single active centre, but no conclusive distinction can at present be afforded between these hypotheses.

We are grateful to Professor Stanley Peat, F.R.S., for his interest, and to the Department of Scientific and Industrial Research for a maintenance grant (to J.M.B., 1949–52) during the tenure of which this work was carried out.

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The Mechanism of Carbohydrase Action

4. THE MECHANISM OF D-ENZYME ACTION

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D-Enzyme is a disproportionating transglycosylase found in potatoes (Peat, Whelan & Rees, 1956). It acts on maltodextrins by transferring α-1:4-linkages (Peat, Whelan & Kroll, 1956). The smallest donor substrate on which it exerts a rapid action is maltotriose. There is some doubt whether it has any action at all on the lower homologue, maltose. Maltose can nevertheless act as an acceptor for transferred maltodextrinyl units, as can glucose and several mono- and di-saccharides, and also monosaccharide derivatives such as methyl α-glucoside (Peat, Whelan & Rees, 1956; Peat, Whelan & Jones, 1957). There are four ways in which a donor and acceptor substrate can be considered to react under the influence of D-enzyme. In the case where glucose (G) is the acceptor the reaction with the donor substrate (N–R) can be formulated as follows (R is the part of the molecule containing the free reducing-end group):

$$N\text{–}R + G \rightleftharpoons N\text{–}G + R, \quad (1)$$

$$N\text{–}R + G \rightleftharpoons R\text{–}G + N, \quad (2)$$

$$N\text{–}R + G \rightleftharpoons G\text{–}N + R, \quad (3)$$

$$N\text{–}R + G \rightleftharpoons G\text{–}R + N. \quad (4)$$

Peat *et al.* (1957) have already shown that reactions (3) and (4) do not take place, but their work left undecided the question whether the reaction followed eqn. (1) or eqn. (2). We now report evidence that D-enzyme acts according to eqn. (1).

EXPERIMENTAL AND RESULTS

D-Enzyme. The enzyme was prepared as by Peat *et al.* (1957).

Preparation of maltodextrins. A digest containing glucose (25·7 mg.), D-enzyme (50 mg.) and solubilized potato starch (20 mg. as free glucose; Hanes & Cattle, 1938) in 2 ml. was incubated under toluene for 17·5 hr. at 30°, and the enzyme was then inactivated by heat. The mixture was centrifuged, and portions of the supernatant solution were subjected to chromatography in propanol–ethyl acetate–water (6:1:3, by vol.) on Whatman no. 3MM paper (18½ in. × 22½ in.) which had been soaked overnight in 1% acetic acid and then washed with water before drying. A portion (0·5 ml.) was spread along a 12 cm. line on one paper and a second portion (0·85 ml.) along an 18 cm. line on another. The papers were irrigated for 24 and 48 hr. respectively, control spots of a mixture of glucose and maltodextrins being used to locate the positions of the digest components by spraying with benzidine–trichloroacetic acid (Bacon & Edelman, 1951). The first paper was used to determine glucose, maltose and maltotriose, and the second to determine maltose–maltohexaose. The two papers were used because glucose and the maltodextrins up to maltohexaose could not be adequately separated on one paper. The digest components were cut out as strips from the papers and eluted with 1·5N-H_2SO_4 (25 ml.) by stirring in a beaker. The solution was filtered through sintered glass, and a 20 ml. portion was heated in a boiling-water bath for 2 hr., cooled, and neutralized with a predetermined volume of approx. 5N-NaOH. Glucose itself was not heated with acid. The reducing powers (as glucose) of 10 ml. portions were then determined with Somogyi (1945) reagent and the yield of each sugar was calculated, correction being made for loss of glucose on heating in acid (Pirt & Whelan, 1951). The yields, expressed as free glucose, were: glucose, 22·6 mg.; maltose, 2·79 mg.; malto-triose, 10·3 mg.; -tetraose, 5·13 mg.; -pentaose, 3·86 mg.; -hexaose, 2·36 mg. The total yield was 47·0 mg., as compared with 45·7 mg. of starch and glucose originally present.

Preparation of [^{14}C]*glucose.* [^{14}C]Glucose was prepared by acidic hydrolysis of uniformly labelled starch (0·1 mc; Radiochemical Centre, Amersham, Bucks). The experimental conditions, devised in experiments with inactive material, were as follows. The starch (about 100 mg.) was heated in 0·5N-H_2SO_4 (10 ml.) for 6 hr. at 100°, cooled and neutralized (NaOH). The solution was passed through charcoal–Celite (1:1, 20 g.) to remove oligosaccharides and then through Biodeminrolit resin (15 g., The Permutit Co. Ltd., London, W. 4) to remove salts. The resin had previously been stored in aqueous suspension overnight under a slight pressure of CO_2. This treatment is essential, otherwise serious losses of sugar will result (Woolf, 1953). The sugar was completely eluted by passing a total of 500 ml. of water successively through the two columns, most of it being in the fraction 250–500 ml. The recovery was 96% of chromatographically pure glucose.

Preparation of [^{14}C]*maltodextrins.* The dextrins were prepared by incubating soluble starch (100 mg.), [^{14}C]-glucose (97 mg.) and D-enzyme (200 mg.) in 8 ml. of solution for 17 hr. at 30°, under toluene. After heat-inactivation of the enzyme, the digest was passed successively through charcoal–Celite (to adsorb maltodextrins) and Biodeminrolit resin (to remove buffer salts), as in the preparation of [^{14}C]glucose. The charcoal column was then eluted with 50% (v/v) aqueous ethanol (300 ml.) to desorb the maltodextrins. The eluate was evaporated to dryness under reduced pressure and distributed between three sheets of Whatman no. 3MM paper which were irrigated for 50 hr. in the propanol–ethyl acetate–water solvent. The di-,

tri- and tetra-saccharide fractions were located by monitoring the paper with a Panax 5054 ratemeter (Panax Equipment Ltd., Mitcham, Surrey). The higher saccharides were located by radioautography with Ilford Industrial F X-ray film. The sugars were eluted by perfusing the paper strips with water, as in chromatography, about 20 ml. of solution being collected from each strip. The solutions were evaporated to dryness under reduced pressure and the sugars dissolved in hot 80% (v/v) methanol. The solutions were filtered to remove paper fibre, and re-evaporated.

Position of radioactive labelling in maltotriose

[^{14}C]Maltotriose (16 mg.), which was chromatographically pure, was dissolved in water (0·8 ml.) and sodium borohydride (20 mg., 0·1 ml.) was added. After the solution had stood for 20 hr. at room temperature 3N-H_2SO_4 was added until there was no further effervescence (0·15 ml.). One portion (0·45 ml.) was mixed with 3N-H_2SO_4 (0·056 ml.; final acid normality, 0·33) and heated at 100° for 30 min. in order partly to hydrolyse the maltotri-itol. A second portion was mixed with 3N-H_2SO_4 (0·090 ml.; final acid normality, 0·5) and heated for 4 hr. at 100° to hydrolyse the sugar alcohol completely. Each hydrolysate and the remainder of the maltotri-itol were passed through Biodeminrolit resin (2 g.) to remove electrolyte. The eluates were evaporated to dryness and methanol (5 × 10 ml.) was distilled off the sugar residue in order to remove boric acid (Zill, Khym & Cheniae, 1953), which is not retained by the resin when in the carbonate form. The products were examined by paper chromatography in butanol–pyridine–water (6:4:3, by vol.) (*A*), and in ethyl acetate–acetic acid–saturated aqueous boric acid (9:1:1, by vol.) (*B*) (W. R. Rees & T. R. Reynolds, in preparation). Reducing sugars were located with benzidine–trichloroacetic acid and reducing and non-reducing sugars with either periodate–benzidine (Cifonelli & Smith, 1954) or $AgNO_3$–NaOH (Trevelyan, Procter & Harrison, 1950). The following information, obtained in control experiments, will explain how the results of chromatography were obtained and interpreted. In solvent *A* there is no perceptible separation of maltotriose from maltotri-itol, or of maltose from maltitol. Glucose and sorbitol separate sufficiently well for them to be recognized as discrete spots, sorbitol having the lower R_F value. The R_F values of glucose, maltose and maltotriose are 0·37, 0·26 and 0·19 respectively, so that it was convenient to examine the partial hydrolysate of maltotri-itol in this solvent. In solvent *B*, however, the movement of the di- and tri-saccharides and their alcohols is so slow relative to glucose and sorbitol that the last two substances are eluted from the paper before the oligosaccharides have moved sufficiently from the origin. The great advantage of solvent *B* is that it achieves a clear-cut separation of a sugar and its corresponding alcohol. The following are typical results. After 22 hr. separation: sorbitol, 31·5 cm. movement; glucose 16·4 cm. After 36 hr.: maltitol, 8·5 cm.; maltose, 5·5 cm. After 110 hr.: maltotri-itol, 9·5 cm.; maltotriose, 7·3 cm. The solvent mixture is very volatile, and, while the relative rates of movement are reproducible, the absolute rates are not. When the two solvents were used the following observations were made. (*a*) The maltotri-itol was chromatographically pure (solvents *A* and *B*), and therefore reduction by borohydride was complete (cf. Whelan & Morgan, 1955). (*b*) The partial hydrolysate contained radioactive material in the regions corresponding to maltotriose + tri-itol, maltose + maltitol and sorbitol; glucose was inactive (solvent *A*). There was no sign of any disaccharide fraction other than maltose + maltitol. The original trisaccharide therefore contained only 1:4-linked glucose units. (*c*) The portion that had been hydrolysed for 4 hr. contained sorbitol (radioactive), glucose (inactive) and a trace of radioactive material in the disaccharide region (solvent *B*), which either represented incompletely hydrolysed material or was an acid-reversion product.

A portion of this same hydrolysate, representing about 0·1 mg. of original maltotriose, was fractionated in solvent *B*. The zones corresponding to glucose and sorbitol were cut out and eluted with water. After evaporation to dryness the boric acid was removed as before and the residue dissolved in water (1 ml.). Portions (0·35 ml. each) were transfered to polythene planchets (1 cm. diam.) and the radioactivity was measured with an Ekco N530C scaler (Ekco Electronics Ltd., Southend-on-Sea). The radioactivity of the sorbitol, expressed as counts/100 sec., corrected for background and coincidence, was 871. That in the glucose fraction was 2. These results, taken with the fact that there was twice as much glucose present as sorbitol, show that the radioactivity in the original maltotriose was centred exclusively in the reducing-end glucose unit.

Position of radioactive labelling in the other maltodextrins

The di-, tetra-, penta- and hexa-saccharide fractions were examined by paper chromatography in solvent *A*. They were chromatographically pure except for the disaccharide fraction, which was resolved into two zones, one moving with maltose and the other (*X*) with isomaltose. The radioactivity associated with the zones was in the approximate ratio 4:1. The two components were separated by chromatography of the whole disaccharide fraction on thick filter paper. Each of the sugars maltose, *X*, malto-tetraose, -pentaose and -hexaose was then examined for the position of radioactive labelling, as for maltotriose, i.e. by the sequence of operations

(i) reduction with borohydride, (ii) acidic hydrolysis, (iii) deionization and removal of boric acid as methyl borate, (iv) chromatographic separation in solvent *B*. The glucose and sorbitol zones were not, however, eluted, the radioactivity being detected qualitatively by radioautography. With maltose, maltotetraose, -pentaose and -hexaose the radioactivity was present solely in the derived sorbitol. With disaccharide *X*, however, both the glucose and sorbitol zones were radioactive although the latter zone appeared to have several times more radioactivity than the glucose.

DISCUSSION

The reaction between starch and glucose, catalysed by D-enzyme, leads to the production of maltodextrins in excellent yields. From the total amount of maltodextrins obtained it would seem that almost the whole of the starch had been utilized in their production. This could not happen unless the α-1:6-branch linkages of the amylopectin component, representing about 75% of the starch, had been broken. It was expected that this would take place since the D-enzyme preparation is known also to contain the debranching R-enzyme (Peat, Whelan & Hobson, 1951), the presence of which can be recognized by the increase in intensity of iodine stain which takes place when the D-enzyme preparation is incubated with starch alone. The method of preparing maltodextrins as now described is an advance on a previously published method (Peat, Whelan & Kroll, 1956) since this latter technique involved the prior production of maltotriose, by α-amylolysis of starch, for use as the substrate from which D-enzyme produced other maltodextrins.

When starch and [^{14}C]glucose are incubated with D-enzyme the maltodextrin products are labelled with radioactivity exclusively in the reducing-end glucose unit. Such dextrins have previously been prepared by the reaction between [^{14}C]glucose and cyclohexa-amylose, catalysed by *Bacillus macerans* amylase (Pazur, 1955; Pazur & Budovich, 1956). The donor substrate and enzyme used in the present work are much more accessible.

End-labelled maltodextrins provide a means of studying the pattern of breakdown effected by starch-metabolizing enzymes. They have also been used to determine the effect of position within the maltodextrin molecule on the rate at which acid hydrolyses α-1:4-glucosidic linkages (G. J. Walker & W. J. Whelan, in preparation). As regards D-enzyme itself, the finding that the maltodextrin products of its action are labelled only at the reducing end proves that it acts according to eqn. (1) and not in the manner expressed by eqns. (2), (3) or (4). That eqn. (3) or (4) cannot hold is self-evident and confirms the earlier findings of Peat *et al.* (1957). With eqn. (2) a single transfer between an inactive maltodextrin substrate and radioactive glucose would yield a polymer labelled only at the reducing end, but a second participation of this molecule in such a transfer would cause radioactivity to appear elsewhere in the molecule (eqns. 2*a*, 2*b*):

$$C_x—G_y—G_{z,R} + G^* \rightleftharpoons G_y—G_z—G^*_R + G_x, \quad (2a)$$

$$G_y—G_z—G_R + G^* \rightleftharpoons G_z—G^*—G^*_R + G_y, \quad (2b)$$

($G^* = [^{14}C]$glucoses; G_R denotes reducing-end group).

The disaccharide products of the reaction between starch and [^{14}C]glucose call for comment. One of these was maltose, labelled only in the reducing-end unit. It is doubtful whether maltose is a true product of D-enzyme action. The amounts of maltose formed when D-enzyme acts on other maltodextrins or, as in the present case, on starch plus glucose, are always small by comparison with other products, and it may well be that traces of amylolytic activity in the enzyme preparation are responsible. It is not yet known what is the structure of the second disaccharide (*X*), which was formed in even smaller amounts than maltose and has not previously been detected. Its paper-chromatographic and -ionophoretic behaviour was like that of isomaltose and gentiobiose. The fact that it was radioactive showed it to be a product of an enzyme-catalysed reaction and not a contaminant of the enzyme preparation. That it was labelled in both sugar units, although unequally, points to a direct polymerization of glucose, such as has already been observed with α- and β-glucosidases (Peat, Whelan & Hinson, 1952, 1955). It has now been shown (unpublished experiments) that the D-enzyme preparation does in fact slowly catalyse the dimerization of glucose and the identification of the product is in progress.

SUMMARY

1. The reaction between starch and glucose, catalysed by D-enzyme, is a convenient means of preparing maltodextrins.

2. When [^{14}C]glucose is used in this reaction the maltodextrin products are radioactive, the activity being confined solely to the reducing-end glucose unit.

3. The proof of the position of radioactive labelling in the maltodextrins has enabled a precise formulation to be given to the manner in which D-enzyme transfers glycosyl radicals between donor and acceptor substrates.

4. An unidentified disaccharide is a minor product of the reaction between starch, glucose and D-enzyme, and may have arisen by enzymic polymerization of glucose.

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Toxicity of Autoxidized Squalene and Linoleic Acid, and of Simpler Peroxides, in Relation to Toxicity of Radiation

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Dam & Granados (1945) found that in rats deficient in vitamin E there was an increase in organic peroxide-like material (hereinafter abbreviated to 'organic peroxide'). They suggested that it resulted from the well-known chain reaction by which unsaturated fats, rubber, etc. 'autoxidize' in air to form peroxides. Though this interpretation is not the only possible one it was the first plausible indication that autoxidation could occur *in vivo* as well as *in vitro*, and was consistent with the known 'antioxidant' properties of vitamin E (for reviews see Mattill, 1947; Holman, 1954). According to the accepted mechanism chain autoxidation is initiated by free radicals having a high content of chemical free energy, and may be terminated by formation of low-energy free radicals such as semiquinones, as happens in the presence of vitamin E and other antioxidants. In absence of antioxidants one high-energy free radical can give rise to several hundred molecules of organic peroxide before the chain is terminated by mutual reaction of two free radicals. In many chemical systems the ions produced by 'ionizing radiations' (X-rays, etc.) give rise to high-energy free radicals, e.g. OH from water (Weiss, 1944). Thus X-rays would be expected to induce autoxidation, and Mead (1952) has found *in vitro* that they do, though the conditions seem to be critical because V. J. Horgan & J. St L. Philpot (unpublished work) failed to repeat the experiment. The question whether X-rays induce autoxidation *in vivo* as well as *in vitro* was provisionally answered in the affirmative by Horgan & Philpot (1953, 1954*a*, *b*), who extracted mice that had received a lethal dose (950 r.) of X-rays. With various methods of peroxide estimation, they sometimes found a significant increase ($P < 0{\cdot}01$; apparent mean chain length about 80) as compared with unirradiated mice. The technique was, however, unreliable and is still being developed. Dubouloz & Dumas (1954) found that in rat skin, 0·1 mm. thick, 5000 r. of X-rays produced 2×10^{-9} and 20 000 r. produced $1{\cdot}3 \times 10^{-8}$ mole/cm.^{2} of peroxide (for references from 1950 onwards see Dubouloz & Dumas, 1954). These figures correspond to mean chain lengths of about 14 and 23 respectively; but it is not clear what fraction of this peroxide was in the living part of the skin. Thus there is some evidence that X-rays can initiate autoxidation *in vivo*, though the observed peroxide could be formed in some other way.

There are not many substances in living matter with a structure suitable for chain autoxidation. The most suitable are the 'methylene-interrupted' [m, $(m+3n)$ diene and polyene] fatty acids, linoleic, linolenic and arachidonic, i.e. the essential fatty acids. Other possibilities are the monoenes, notably oleic acid, the carotenoids [conjugated, i.e. m, $(m+2n)$ polyenes] and squalene [m, $(m+4n)$ polyene]. Though all these substances might be

invoked to explain the observations, Horgan & Philpot tried to narrow the field on the basis of an unexpected observation that roughly half the peroxide in their extracts seemed to be less reactive than typical hydroperoxides and behaved more like disubstituted peroxides. This suggested that the substance predominantly concerned might be squalene, whose primary autoxidation product was shown by Bolland & Hughes (1949) to contain one hydroperoxide and one disubstituted (cyclic) peroxide group, as opposed to the primary product from oleic or linoleic acids, which has only a hydroperoxide group (Farmer & Sutton, 1943; Swift, Dollear & O'Connor, 1946; Bolland & Koch, 1945). With linoleic acid, however, the double bonds are shifted into the conjugated position during autoxidation and can then apparently add a second molecule of oxygen to form a cyclic or polymeric peroxide group (cf. Bergström, Blomstrand & Laurell, 1950). The work of Bergström *et al.* was not known to Horgan & Philpot, and as they could find no data concerning a second molecule of oxygen they ignored the possibility. The high toxicity of radiation might be explained if squalene diperoxide, formed by autoxidation, could act as an antimetabolite in blocking cholesterol synthesis, for which squalene was shown to be an intermediate by Langdon & Bloch (1953). If a specific blocking mechanism like this existed squalene diperoxide might be far more toxic than ordinary peroxides. The testing of this possibility was the original purpose of the work described below.

At the same time, apart from the comparison with squalene, data were needed on the toxicity of injected linoleic acid hydroperoxide and peroxides in general. It is already known (Wyss, Clark, Haas & Stone, 1948; Dickey, Cleland & Lotz, 1949; Ross, 1950; Loveless, 1951) that peroxides are mutagenic; but little is known as to their toxicity on injection. Apart from toxicity the autoxidation of essential fatty acids, if extensive, might cause them to be depleted (cf. Sinclair, 1956). In support of this view Cheng, Kryder, Bergquist & Deuel (1952), following earlier work of Decker, Fillerup & Mead (1950), showed that the survival time of chronically X-irradiated rats was less on a fat-free diet than on the same plus methyl linoleate. If radiation toxicity is due to autoxidation of essential fatty acids, giving either toxic products or deficiency, it is surprising, though possibly explicable, that vitamin E fails to protect (Herve & Bacq, 1949; Horgan & Philpot, 1954*b*; Haley, McCulloch & McCormick, 1954).

Our toxicity tests on peroxides of squalene and linoleic acid required a knowledge of their concentration, since we did not separate them from the parent substances. The occasion was used to study the behaviour of autoxidized pure linoleic acid towards the latest form of our reagent, and to compare it directly with autoxidized squalene. Previously we had available only some doubtfully pure ethyl oleate.

EXPERIMENTAL

Estimation of peroxides. The method of peroxide estimation used here has been developed primarily for butanol extracts of mice, where oxygen must be rigorously excluded to avoid possible interference by aerobic catalysts such as parahaematins, which might be present in the extracts. When strictly anaerobic, most reagents fail to react even with peroxide; but leuco-brilliant cresyl blue in a suitable buffer is an exception. Under our present conditions it can distinguish two types of reactivity according to whether a cuprous salt is needed ('type 2') or not ('type 1'). Further differentiation can be made according to the rate of reaction, and also according to the rate of destruction of peroxide by the buffer in absence of cuprous salt. This classification is purely empirical and has no simple relation to structure or to the division into 'reactive' and 'less reactive' made on a temperature basis by our earlier reagents; but it is nevertheless useful analytically.

The procedure is as follows: 1·4 ml. of about 5×10^{-5}M-peroxide in butanol is placed in a 2 ml. stoppered spectrophotometric cell and oxygen-free nitrogen is bubbled through the solution for 4 min. The cell is mounted beside a similar control cell, at the bottom of a small glass hood with an opening at the top allowing additions to be made against a flow of nitrogen. Through the opening 0·5 ml. of saturated brilliant cresyl blue (approx. 8×10^{-4}M) in butanol buffered with 0·5M-lutidine plus 0·25M-acetic acid is run into the cell from a burette fitted with a U-tube containing a reducing column of cadmium filings. Any failure of anaerobic conditions is shown by blue replacing the yellow of the leuco-dye in the control cell, but this is rare. The absorption at 620 mμ in this cell and in the test cell after various lengths of time at room temperature is measured in a spectrophotometer after inserting the stoppers through the hood and removing the cells. Under these conditions $\log I_0/I$ for a 5 mm. thickness is 1·0 for every $6{\cdot}95 \times 10^{-5}$ mole of reoxidized brilliant cresyl blue. The above procedure measures the extent of the type 1 reaction. For the total reaction the cuprous salt is added before the buffered leuco-dye, and the extent of the type 2 reaction is estimated from the total by difference. Destruction by buffer is tested by adding the cuprous salt at various times after the buffer. Tests with mixtures of known peroxides failed to reveal interaction, so that one type can apparently be estimated in the presence of another.

The cuprous salt (substantially lithium cuprobromide) is prepared by passing 0·02 ml. of 5×10^{-3}M-cupric chloride (hydrated) plus 0·1M-lithium bromide plus 0·02M-lithium acetate in butanol through a burette fitted with a reducing column of copper powder.

Materials

Brilliant cresyl blue

This was freed from the red impurity previously described (Horgan & Philpot, 1954*a*), by Soxhlet extraction with *n*-butyl acetate.

2:6-*Lutidine*

This was distilled before use and stored under nitrogen.

n-Butanol

This was refluxed for an hour over cadmium filings plus lithium bromide to remove peroxide, and distilled. It was used within 24 hr.

Disuccinoyl peroxide

This was kindly provided by Dr Latarjet.

Other peroxides

These were made by approximately standard methods (cf. Tobolsky & Mesrobian, 1954), except *tert.*-butyl hydroperoxide and dibenzoyl peroxide, which were bought. The solid ones were recrystallized and the liquid ones redistilled shortly before use.

Hydrogen peroxide

This was standardized by the use of an iodometric technique (cf. Vogel, 1951).

Squalene

This was a commercial product which we purified by passage through an alumina column. A highly purified specimen kindly provided by Dr Heller behaved similarly on autoxidation but the toxicity of its product has not yet been measured.

Linoleic acid and methyl linoleate

These were highly purified products kindly provided at cost price by the Hormel Foundation (Austin, Minn., U.S.A.).

Autoxidation. The squalene and linoleic acid were originally autoxidized by slowly bubbling air through them without a catalyst at room temperature (about 20°). In later experiments with linoleic acid and methyl linoleate a roller-tube was used for aeration. Some experiments with linoleic acid were done also with a thin film spread on glass, diffusion of air being relied upon. Most experiments were done in a slow stream of air (from the compressed-air line), dried with silica gel and magnesium perchlorate ('anhydrone') and filtered through glass wool.

Animals. Three strains of mice were used: CBA, hairless albino and R [a cross between PCT female and (C3H × 101) male]. R mice were all 3–3½ months old and had a mean wt. of 22·7 ± S.E.M. 0·37 g. (37 mice). They were fed on a standard cube ration.

Toxicity determinations. Solutions of the peroxides were injected intraperitoneally into female mice in volumes of 0·1–0·4 ml. Disuccinoyl peroxide was dissolved in water. The others, being insoluble in water, were dissolved in ethyl palmitate or in squalene (for comparison with squalene diperoxide, which was prepared in squalene by autoxidation). The doses of *tert.*-butyl hydroperoxide, autoxidized linoleic acid, methyl linoleate and squalene were determined by peroxide estimation; those of benzoyl peroxide, hydroxy*cyclo*hexyl hydroperoxide, tetralin hydroperoxide and disuccinoyl peroxide were determined by weight and by peroxide estimation; and those of di-*tert.*-butyl peroxide by weight alone. The doses were chosen by trial so as to approach the LD_{50} with as few mice as possible. A number of mice were then injected with doses near the LD_{50} until it was determined with sufficient accuracy. This procedure sufficed for present purposes instead of a formal probit analysis, because of the sharpness of the dose–response curves. The mice were kept for 30 days after injection and inspected twice daily for the first two days, then daily.

RESULTS

Types of reactivity of peroxides

Table 1 gives the percentage of theoretical colour development with known peroxides under the conditions described above for distinguishing two types of reaction. The figure for hydrogen peroxide assumes that the iodimetric method gives 100 % reaction. The other figures assume that the substances were pure. The present form of reagent, applied to extracts of mice, shows the presence of both types as expected from previous results, but we have not studied them properly.

The behaviour of di-*tert.*-butyl peroxide was puzzling and needs further study. The first specimen of this that we made gave a type 2 reaction to the extent of half an equivalent with no destruction by buffer. A mixture with *tert.*-butyl hydroperoxide (type 1) behaved additively, i.e. the total colour was equivalent to the hydroperoxide in the absence of cuprous salt and to this plus half the di-*tert.*-butyl peroxide in the presence of cuprous salt. After storage for a month at 3° the di-*tert.*-butyl peroxide failed to react under any conditions, although the substance is said to be exceptionally stable. A second specimen was prepared and behaved exactly like the first, reacting when fresh but not after storage. A third specimen failed to react even when fresh, although it and the other two all boiled at 111°, which is the accepted b.p. The third specimen had m.p. −37° (accepted −40°). Neither the second nor the third specimen reacted with iodide even when fresh, showing that hydroperoxide was absent. The first specimen was not tested with iodide. The material used for toxicity tests was specimen 2, freshly prepared. Diethyl peroxide has a slow type 2 reactivity. In the presence of cuprous salt it oxidizes the leuco dye with a half-reaction time of about 35 min. at 23°, and if the buffer is added 5 min. before the cuprous salt about 12 % of

Table 1. *Leuco-brilliant cresyl blue reagent tested with various peroxides*

Type 1 reaction in the absence, type 2 in the presence, of cuprous salt.

Peroxide	Percentage of theoretical colour development: Type 1	Type 2
Disuccinoyl peroxide	100–101	0
1-Hydroxy*cyclo*hexyl 1-hydroperoxide	94	—
tert.-Butyl hydroperoxide	97	—
Hydrogen peroxide	91	—
Tetralin hydroperoxide	1–11	92–99
Diethyl peroxide	2	40 (99 in 90 min.)
Di-*tert.*-butyl peroxide	0	0 or 47 (see text)

the peroxide is destroyed. With tetralin hydroperoxide, destruction by buffer is complete in a few minutes, but nevertheless cuprous salt enables it to oxidize the leuco dye before it is destroyed.

Autoxidation of squalene and linoleic acid

Fig. 1 shows a log plot of the peroxide contents of squalene and linoleic acid during autoxidation at room temperature in a bubbling tube with untreated air from the compressed-air line. The linoleic acid had been imperfectly anaerobic at 0° for a week after opening, which may have shortened the induction period. The straight lines (except for the first squalene point which was too low for accuracy) show that the kinetics are ideally autocatalytic, in accordance with the slow branched-chain reaction mentioned in the introduction, with no complications such as change in length of the fast linear-reaction chains. Each substance has two straight lines, one for type 1 peroxide reaction only and the other for total reaction. The difference in ordinates (0·3, i.e. approx. log 2) shows that the total is apparently almost exactly twice the type 1 in both cases. Thus there seem to be two types of peroxide group present in both cases, in equivalent amounts if they both give equal yields of oxidized dye. Autoxidation was still continuing at the steadily increasing rate when the products were used for toxicity tests. The total molarity of peroxide groups was then 0·92 for linoleic acid and 0·24 for squalene.

Unfortunately for simplicity, we repeated the linoleic acid autoxidation primarily in order to measure the oxygen uptake and thereby to verify that a second molecule of oxygen was combining to form a cyclic or polymeric peroxide group, which would explain the existence of two types of peroxide reaction as with squalene. The oxygen uptake was measured by weighing on a microbalance, with a light shallow-glass dish supported by three thin legs on a second dish, to avoid loss by creeping, as reaction vessel. The result is shown as a linear plot in Fig. 2. Clearly only one molecule of oxygen is taken up, along a smooth S-shaped curve; but more surprising is the behaviour of the peroxide. Through the major part of the oxygen uptake only the type 1 peroxide reaction is perceptible; but near the end there is a sudden replacement of half of the type 1 reaction by its equivalent of type 2, without further uptake of oxygen. This change seemed, qualitatively, to be accompanied by a sudden increase in viscosity. The conjugated diene was estimated from the absorption at 231 mμ, and is plotted in the same figure. In later repetitions with the above reaction vessel (called a 'cake-stand' from its appearance), with a roller-tube and with the original bubbler-tube, the behaviour was roughly like that of Fig. 2, but the sudden loss of type 1 peroxide reaction occurred irregularly at one or more earlier stages, and was not necessarily accompanied by an equivalent increase in type 2 reaction. Duplicate assays never disagreed by more than 10 %, which is small compared with the irregularities in question. In one roller-tube experiment the type 2 reaction was in the opposite direction, i.e. less colour was developed in the presence of cuprous salt than in its absence. This was shown to be due to a substance that could reduce brilliant cresyl blue in the presence of the cuprous salt; thus the measured type 2 reaction is

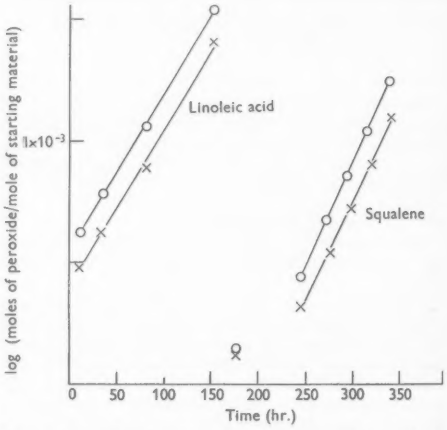

Fig. 1. Autoxidation of linoleic acid and squalene with air in bubbler-tube (moles of peroxide/mole of starting material). ×, Peroxide determined in the absence of Cu^+ ion; ○, peroxide determined in present of Cu^+ ion.

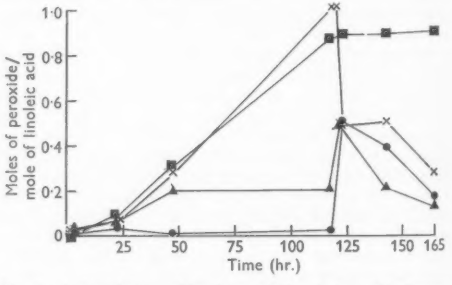

Fig. 2. Autoxidation of linoleic acid in air in a thin layer, unstirred (moles of peroxide/mole of starting material). ■, Oxygen absorbed (gravimetric); ×, peroxide determined in the absence of Cu^+ ion; ●, peroxide determined in the presence of Cu^+ ion minus peroxide determined in absence of Cu^+ ion; ▲, conjugated diene estimated from absorption at 231 mμ. At 115 hr. the viscosity seemed to increase suddenly.

the net result of two opposing reactions, either of which can predominate. A typical roller-tube experiment where the net type 2 reaction was positive is illustrated in Fig. 3, where in addition to the measurements already described the absorption developed by thiobarbituric acid at 520 mμ (cf. Bernheim, Bernheim & Wilbur, 1948) is shown. In the butanol solvent used by us thiobarbituric acid reacts with autoxidized linoleic acid but fails to react with the simpler peroxides. The colour is orange if developed in air, or red in nitrogen turning to orange on admitting air. The main absorption peak in nitrogen is at about 540 mμ and in air at 460 mμ.

Toxicities

Fig. 4 shows dose–mortality curves for R mice and Table 2 shows the estimated LD_{50} for all the peroxides except diethyl peroxide. The latter was originally intended as a general model of disubstituted peroxides, which might help in estimating the importance of the cyclic or polymeric peroxide groups in the diperoxides of linoleic acid and squalene. It was abandoned for fear that its volatility might give misleading results, though two mice given 460 μmoles both died. Later we hoped that di-*tert.*-butyl peroxide would be a more suitable model, but it is less toxic despite its lower volatility. The chief points to note in Table 2 are the normal toxicity of autoxidized squalene and methyl linoleate and the high toxicity of autoxidized linoleic acid as compared with the other peroxides. The three strains of mice are about equally sensitive, except that hairless-albino mice seem to be more sensitive than the others to disuccinoyl peroxide.

It was hoped that injected peroxides might give the delayed deaths characteristic of radiation toxicity, but the delay, though perceptible, is much less. Of 275 R mice that died after peroxide treatment 188 died on the first day, 50 on the second and 34 on the third to fifth day. Only three deaths occurred later than this (on the 11th–14th days). Two of these occurred with autoxidized linoleic acid and one with di-*tert.*-butyl peroxide. For comparison, of 177 R mice that died in various radiation experiments after 850–1000r. of whole-body X-irradiation, none died in the first 3 days, 38 died between 4 and 8 days, 121 died between 9 and 16 days and 15 died between 17 and 30 days after treatment.

Table 3 summarizes the results of control tests on solvents and substances related to the peroxides in various ways, and shows that the toxicities in Table 2 are mainly due to the peroxide groups themselves. It is unlikely that the toxicities of the autoxidized samples of squalene and methyl linoleate were due to unautoxidized material present, since injection of unautoxidized materials in doses several times higher than the LD_{50} doses did not cause any deaths. There were some deaths with higher doses of unautoxidized linoleic acid, acting possibly as an anionic detergent, but Table 3 shows that the acid is far less toxic than its autoxidation product. It is also unlikely that material other than peroxide is responsible for the toxicity of autoxidized linoleic acid. Thus Fig. 4 (*a*) and Table 2 show that four samples of linoleic acid at different stages of autoxidation (and therefore differing widely in their content of peroxide), unchanged linoleic acid and decomposition products all have the same toxicity on a peroxide basis.

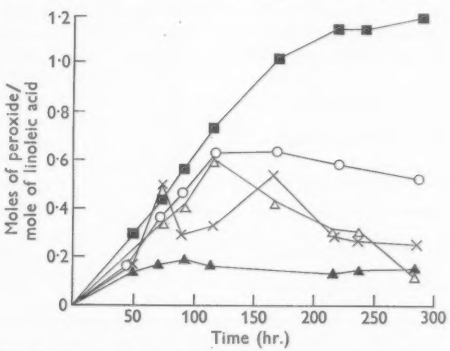

Fig. 3. Autoxidation of linoleic acid in roller-tube in air (moles of peroxide/mole of starting material). ■, Oxygen absorbed (gravimetric); ×, peroxide determined in the absence of Cu^+ ion; ○, peroxide determined in the presence of Cu^+ ion; ▲, conjugated diene estimated from absorption at 231 mμ; △, extinctions/10^{-5} mole of starting material (1 cm., 520 mμ) developed by thiobarbituric acid.

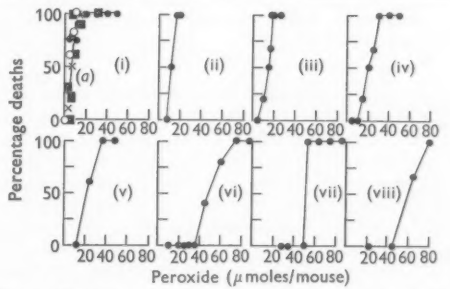

Fig. 4. Toxicities of various peroxides. Curves were obtained with R mice. For further details see Table 2. Curve (*a*) is from four samples of autoxidized linoleic acid; ●, sample I; ○, sample II; ×, sample III; ■, sample III*a* (see footnote to Table 2). (i) Autoxidized linoleic acid; (ii) disuccinoyl peroxide; (iii) hydroxy-*cyclo*hexyl hydroperoxide; (iv) benzoyl peroxide; (v) autoxidized squalene; (vi) autoxidized methyl linoleate; (vii) tetralin hydroperoxide; (viii) *tert.*-butyl hydroperoxide.

Table 2. *Toxicity of peroxides in three strains of mice*

For method see text.

Compound	Strain of mouse	Solvent	LD_{50}. Peroxide (μmoles/mouse)	Original autoxidizable substance (μmoles/mouse)	No. of mice used in test
Autoxidized linoleic acid*					
Sample I	R	Ethyl palmitate	<4	<13·0	28
Sample II	R	Ethyl palmitate	6	12·8	35
Sample III	R	Ethyl palmitate	7	10·8	47
Sample III*a*	R	Ethyl palmitate	6–8	17·6–23·5	80
Disuccinoyl peroxide	R	Water	12	—	16
	CBA	Water	10–12	—	20
	Hairless albino	Water	4–6	—	29
1-Hydroxy*cyclo*hexyl hydroperoxide	R	Ethyl palmitate	15	—	37
	CBA	Ethyl palmitate	18–20	—	16
	CBA	Squalene	15–16	—	20
Dibenzoyl peroxide	R	Ethyl palmitate	20	—	47
	CBA	Ethyl palmitate	20	—	16
Autoxidized squalene*	R	Squalene	22	162	29
	Hairless albino	Squalene	50	—	10
Autoxidized methyl linoleate*	R	Ethyl palmitate	45–60	69–92	70
Tetralin hydroperoxide	R	Ethyl palmitate	52	—	32
	CBA	Ethyl palmitate	36	—	16
tert.-Butyl hydroperoxide	R	Ethyl palmitate	58	—	24
	CBA	Ethyl palmitate	60–70	—	8
Di-*tert.*-butyl peroxide†	R	Ethyl palmitate	1080	—	39

* The three samples of linoleic acid used had autoxidized to the extent of 0·31, 0·47 and 0·65 mole of total peroxide/mole of original linoleic acid respectively. Sample III*a* was obtained by allowing sample III to stand in air for a week until its total peroxide had fallen to 0·34 mole of total peroxide/mole of original linoleic acid. The samples of squalene and methyl linoleate were autoxidized to the extent of 0·14 and 0·65 mole of total peroxide/mole of starting material respectively.

† Dose was calculated from the weight of injected material and not from peroxide estimations.

Table 3. *Control tests of toxicities of solvents and compounds related to peroxides*

All experiments were on female R mice. See Table 2 for toxicities of related peroxides.

Compound	Dose (μmoles/mouse)	No. of mice treated	No. of deaths
Linoleic acid	32·5	10	0
	65·0	5	0
	89·5	10	2*
	195	5	1
Methyl linoleate	78	5	0
	156	5	0
Squalene	635	5	0
*cyclo*Hexanol	20	5	0
tert.-Butanol	80	5	0
Ethyl palmitate (solvent)	0·6 ml.	5	0

* One of these deaths was probably not due to the treatment.

Table 4. *Toxicities of butyl halides on injection into CBA-strain mice*

Compound	LD_{50} (μmoles/mouse)	No. of mice used
tert.-Butyl chloride	600–700	15
n-Butyl chloride	100–150	16
tert.-Butyl bromide	250–300	11
n-Butyl bromide	100–200	19

Table 4 contains data on some butyl halides, which were tested for comparison because it was thought that the peroxides might be acting in a manner roughly analogous to that of alkylating agents, i.e. by chemical substitution. Thus *tert.*-butyl hydroperoxide might be comparable in its effects with *tert.*-butyl chloride or bromide. In fact the butyl halides have very low toxicity, including *tert.*-butyl chloride, which reacts like mustard gas by the 'S_N1' mechanism (Hughes, 1935). This agrees with the usual experience that monofunctional alkylating agents have only slight biological activity (cf. Loveless & Revell, 1949; Loveless, 1951).

DISCUSSION

Four points have emerged during the present work which suggest that there is no need to invoke squalene rather than linoleic acid in attempting to explain radiation toxicity in terms of autoxidation. These are as follows.

(1) The best reagent that we know so far is unable to distinguish one of our samples of autoxidized linoleic acid from autoxidized squalene, at any stage in the autoxidation. Both products give the two types of peroxide reaction to approximately

equal extents, though in other samples of autoxidized linoleic acid the type 1 reaction predominates and the type 2 reaction may even be negative.

(2) Bergström *et al.* (1950) have shown clearly that under some conditions the addition of a second molecule of oxygen to the conjugated double bonds of linoleic acid hydroperoxide can be comparable in speed with the primary reaction. Their peroxide data suggest, however, that under their conditions (aqueous emulsion at pH 9) the diperoxide decomposed as fast as it was formed. Under our acidic anhydrous conditions of autoxidation (*in vitro*) the diperoxide might easily survive till tested, and it should then behave chemically just like squalene diperoxide, in agreement with our first results. Thus the chief difference in the autoxidation of linoleic acid and squalene may be not in the nature of the product but in the kinetics of its formation, since theoretically the two groups appear simultaneously with squalene and successively with linoleic acid. Our later experiments suggest, however, that the type 2 peroxide reaction can be given by linoleic acid which has been autoxidized under conditions where apparently only one molecule of oxygen is taken up. The abruptness and irreproducibility of the formation of type 2-reacting material, the fact that no extra oxygen is taken up when it is formed, and the fact that it seems to be formed from type 1-reacting material, suggest that it may be formed by some non-oxidative chain reaction such as polymerization of the conjugated double bonds to form a rubber-like polymer, still containing the original hydroperoxide groups but with altered reactivity. The origin of the negative type 2 reactivity observed in one experiment is completely obscure. Until the reactions *in vitro* are clearer and more controllable it is impossible to predict what could happen *in vivo*, but it seems likely that all our analyses of mouse extracts will be explicable in terms of autoxidation of essential fatty acids rather than of squalene.

(3) Squalene diperoxide is no more toxic than several simple peroxides, so that the idea that it might act as a specific antimetabolite receives no support.

(4) On a peroxide basis our specimen of autoxidized linoleic acid was more toxic than any of the other peroxides tried, and the average LD_{50} for the four samples used (0·26 μmole of peroxide groups/g.) was slightly less than the mean increased peroxide extracted by Horgan & Philpot from lethally irradiated, as compared with unirradiated, mice (0·22 μmole/g.). Thus without the need to postulate incomplete extraction by butanol or inefficient distribution of injected peroxide from the peritoneum to sensitive sites there is evidence that radiation produces enough peroxide (if derived mainly from linoleic acid) to account for the death of the animal. However, the evidence is tentative until a thorough study of mouse extracts has been made with the improved reagent. The chief discrepancy in comparing toxicities of peroxides and of radiation is in the time scale, since injected peroxides kill more quickly than radiation. This discrepancy, and the fact that vitamin E fails to protect appreciably against radiation *in vivo*, may perhaps be explicable in terms of a difference in distribution of peroxide. Hard X-rays produce chain-initiating free radicals fairly uniformly throughout the animal, and any unsaturated fats (e.g. in the phosphatides of cell membranes) will be liable to autoxidation except in places where vitamin E is stored (e.g. the fat depots). Injected peroxides may be carried by the blood more efficiently to some sites than to others. Thus equal total doses of injected and radiation-produced peroxide may kill different groups of cells, and the animal may survive for longer when deprived of one group than of the other. For the acutely lethal effects of radiation, the cells concerned are predominantly the haemopoietic and the dividing intestinal ones, which die within 24 hr. With injected peroxide it is not known which cells die first, or whether any die before the animal as a whole.

Thus all the facts known to us are consistent with the hypothesis that radiation toxicity in mammals is due to initiation of chain autoxidation of essential fatty acids producing lethal doses of peroxides in sites not reached by vitamin E.

The fact that autoxidized methyl linoleate is far less toxic than autoxidized linoleic acid was quite unexpected. Possibly the ionized carboxyl group anchors the linoleic acid to some sensitive site such as a cell membrane. We hope soon to measure the toxicity of autoxidized phosphatides containing linoleic acid.

SUMMARY

1. An anaerobic reagent is described, consisting of leuco-brilliant cresyl blue in butanol buffered with lutidine and lutidine acetate, with and without lithium cuprobromide. This shows two types of reaction with various organic peroxides, according to the effect of the cuprobromide, with further differentiation according to kinetics.

2. Both types of reaction were obtained equally with the products of uncatalysed autoxidation of squalene and linoleic acid at room temperature, at all stages observed, but the autoxidation of linoleic acid was highly erratic.

3. The previous finding of two types of peroxide in extracts of X-irradiated mice can now be explained in terms of linoleic acid as well as of squalene.

4. On a basis of total peroxide content, autoxidized linoleic acid, unlike autoxidized squalene, was

more toxic by intraperitoneal injection than any of the simple peroxides with which it was compared. Autoxidized methyl linoleate was less toxic than most of the simple peroxides.

5. The LD_{50} of autoxidized linoleic acid (0·26 μ-mole of peroxide/g.) was only slightly higher than the mean increase of peroxide previously found in mice after 950r. of X-rays (0·22 μmole/g.).

6. Injected peroxides kill more quickly than radiation, but this may be a question of distribution.

7. The evidence is consistent with the view that radiation toxicity is due to initiation of chain autoxidation of essential fatty acids producing lethal doses of peroxides in sites not reached by vitamin E.

We wish to thank Dr John H. Heller for highly purified squalene, Dr R. Laterjet for disuccinoyl peroxide, Mr M. J. Corp for X-radiation, Mr R. T. Beadle and Mr J. W. P. Phelpstead for efficient technical assistance, and the animal-house staff for care of the animals.

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The Enzymic Hydrolysis of Adenosine Triphosphate by Liver Mitochondria

I. ACTIVITIES AT DIFFERENT pH VALUES

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Interest in the hydrolysis of adenosine triphosphate (ATP) by mitochondria was stimulated by the suggestion that it may be due to a reversal and diversion of the reactions which are responsible for the synthesis of ATP during the process of oxidative phosphorylation (Hunter, 1951; Lardy & Wellman, 1953; cf. Lardy & Elvehjem, 1945). When the mitochondria are carefully isolated from liver homogenates in isotonic sucrose solution at 0°, they exhibit little or no hydrolytic activity towards ATP (Kielley & Kielley, 1951). The oxidation of a number of substrates by these mitochondria is obligatorily coupled with the phosphorylation of two or three molecules of adenosine diphosphate (ADP) to ATP for each atom of oxygen consumed. The P:O ratio is decreased and the rate of hydrolysis of added ATP is increased when the structure of the mitochondria is damaged, e.g. by exposure to hypotonic solutions,

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incubations at 25–35° in the absence of substrate or exogenous ATP, addition of Ca^{2+} ions or surface-active agents, and physical disintegration in a Waring Blendor or by freezing and thawing. After any of these treatments the oxidation of a substrate may proceed unimpaired, but the presence of ADP and inorganic phosphate is no longer required in order to obtain the maximum rate of uptake of oxygen. The enzyme responsible for the dephosphorylation of ATP by mitochondria is usually designated as a 'latent ATPase', although it is not considered to be a simple hydrolytic enzyme or even a single enzyme (cf. Lardy & Wellman, 1953; Potter, Siekevitz & Simonson, 1953).

2:4-Dinitrophenol (DNP) and analogous phenols also stimulate the latent ATPase activity of normal mitochondria (Hunter, 1951; Potter & Recknagel, 1951; Lardy & Wellman, 1953; Potter *et al.* 1953) and produce a simultaneous uncoupling of the oxidative phosphorylation (Loomis & Lipmann, 1948). These effects appear to be reversible and can occur without any visible alteration in the mitochondrial structure. Similar results have been reported with a variety of other agents which have been less extensively investigated (cf. Hunter, 1955). It seems probable therefore that the uncoupling of oxidative phosphorylation is in some way related to the activation of the latent ATPase activity and that an investigation of the mechanism of hydrolysis of ATP might yield further information concerning the mechanism of oxidative phosphorylation.

The synthesis of ATP probably occurs at three separate points in the respiratory chain during the process of oxidative phosphorylation (cf. Lehninger, 1954; Slater, 1956; Lardy, 1956; Chance & Williams, 1956). Since the uncoupling of the phosphorylation seems to occur at all three points in the presence of DNP, there is a possibility that three separate enzymes or coenzymes might be involved in the hydrolysis of ATP. On investigating the effect of pH on the ATPase activity of mitochondrial preparations, we found evidence of four separate optimum pH values which possibly represent four different enzyme systems. Each of these activities appeared to be relatively specific for ATP. However, the effect of DNP in activating the latent ATPase activity was restricted to three of these pH values. It is possible therefore that these three values represent the three enzyme systems which might be involved in the oxidative phosphorylation. Preliminary reports of these results have been presented (Myers & Tuynman, 1956; Myers & Slater, 1957*a*).

METHODS

Enzyme preparations. Mitochondria were isolated from liver by the general method of Schneider & Hogeboom (1950) as modified by Hollünger (1955) and Hogeboom (1955). Male Wistar rats weighing about 250 g. were stunned and decapitated; a portion of the liver was immediately removed and chilled in cold 0·25 M-sucrose. The liver was weighed, cut into small pieces and ground for about 30 sec. at low speed in a chilled Potter–Elvehjem homogenizer with 10 ml. of cold 0·25 M-sucrose/g. of liver. The homogenizer used in all of these preparations was fitted with a polytetrafluoroethylene (Teflon) pestle and was obtained commercially from the Arthur H. Thomas Co., Philadelphia, Pa., U.S.A. (cf. Potter, 1955). The liver homogenate was subsequently centrifuged for 5 min. at 800 *g* in a cooled centrifuge to remove nuclei, erythrocytes, intact liver cells and debris; approximately three-quarters of the supernatant fluid was decanted and centrifuged for 10 min. at 6000–7000 *g*. The precipitate of mitochondria was resuspended in sucrose solution with the aid of the Potter–Elvehjem homogenizer and centrifuged for 10 min. at about 18 000 *g*. The fluffy layer was removed from the firmly packed sediment of mitochondria by gently shaking with small amounts of sucrose solution, and the residual mitochondria were again resuspended in sucrose solution with the aid of the homogenizer. All operations were carried out at 0°. This procedure, which was designed to isolate intact mitochondria rather than to recover all of the mitochondria present in the liver, yielded about 20 mg. of mitochondrial protein/g. of liver.

The preparations of sarcosomal fragments (cf. Cleland & Slater, 1953) from horse heart were usually isolated by the standard procedure of Keilin & Hartree (1947), as described by Slater (1949), in which the fragments are precipitated from an extract of the heart muscle by acidification to pH 5·7. Other preparations were precipitated by high-speed centrifuging (Slater, 1949).

Determination of enzyme activities. The method used for determining ATPase activity was based on the results obtained by Lardy & Wellman (1953) and Potter *et al.* (1953). The initial experiments were carried out at pH 7·4 in an isotonic medium containing 0·075 M-KCl, 0·108 M-sucrose, 0·001 M-ethylenediaminetetra-acetate (EDTA) and 0·002 M-ATP, with or without 0·003 M-$MgCl_2$ and with or without 0·0001 M-DNP. The total volume was 1·5 ml. The concentration of ATP is the same as that used by Potter *et al.* (1953); the concentration of DNP is intermediate between that used by Lardy & Wellman (1953) and by Potter *et al.* (1953), and is usually sufficient to produce complete uncoupling of oxidative phosphorylation in the respiratory chain. Potassium chloride is required for maximal stimulation of the latent ATPase activity of normal mitochondria by DNP (Lardy & Wellman, 1953); although this salt does not affect the ATPase activity of aged mitochondria it was included in all experiments for comparative purposes. The concentration of $MgCl_2$ is equal to the molar concentration of ATP (cf. Kielley & Kielley, 1951, 1953; Potter *et al.* 1953) plus the molar concentration of EDTA.

The EDTA was added to remove Ca^{2+} or other contaminating metal ions and appeared to be essential in order to obtain a low ATPase activity, in the absence of DNP, with normal mitochondria. It was not established whether this was due to an action of EDTA on the mitochondria (e.g. by removal of contaminating metals) or was due to metal-ion contamination of our sucrose and 2-amino-2-hydroxymethylpropane-1:3-diol (tris) and possibly the ATP. The first two gave a positive reaction for metal ions with

8-hydroxyquinoline (cf. Chappell & Perry, 1953) and a titrimetric assay (Debney, 1952), which was carried out by Mrs A. J. Haarbrink-Haitsma, showed that the 0·25M-sucrose solutions contained about $2·6 \times 10^{-5}$M-calcium. The other solutions seemed to be free of metal impurities. The inhibition of the respiration of mitochondria by high concentrations of ATP suggested that the ATP might also contain impurities which were firmly bound and therefore not detectable by the hydroxyquinoline method. A concentration of 10^{-4}M-EDTA decreased the ATPase activity, in the absence of DNP, to a low level (Table 1). A concentration of 10^{-3}M-EDTA was used in most experiments.

In the experiments at pH 7·4, described in Table 1, the ATP used as substrate was the only buffer present. Subsequent experiments were carried out at different pH values in the presence of 0·05M-tris buffer. The pH values between 4·5 and 9·5 were obtained by titrating 0·25M-tris with 0·25M-acetic acid. To obtain stable buffers at pH 10–11, the 0·25M-tris was first mixed with an equal volume of aq. 0·25M-NH_3 soln. and subsequently titrated with 0·25M-acetic acid. In the descriptions of the experiments, both types of buffer are referred to as tris buffer. A concentration of 0·05M-tris means that these stock buffer solutions were diluted fivefold in the final reaction mixture. This degree of dilution did not appreciably alter the pH, nor was this affected by the concentration of ATP used. The tris–HCl buffer used in the experiment described in Fig. 4 was prepared by titrating 0·2M-tris with 2N-HCl. The histidine–HCl buffer used in the same experiment was prepared by titrating 0·2M-histidine hydrochloride with 2N-NaOH. Both these buffers were also diluted fivefold in the final reaction mixture. The reaction mixture used in these experiments contained 0·075M-KCl, 0·05M-sucrose, 0·05M-buffer, 0·001M-$MgCl_2$, 0·0006M-EDTA and 0·002M-ATP. The EDTA was usually omitted in experiments with fully activated preparations of aged or fragmented mitochondria.

Unless otherwise stated in the text, the mitochondrial preparations were incubated in the reaction mixture for 15 min. at $20 \pm 1°$. The reaction was stopped by the addition of an equal volume of 10% (w/v) trichloroacetic acid, the protein was centrifuged off and the inorganic phosphate in the supernatant fluid determined by the Fiske–Subbarow method as modified by Sumner (1944).

The concentration of the enzyme preparation was adjusted so that not more than 40% of the ATP was hydrolysed; the same concentration of enzyme was used at all pH values in each experiment. Under these conditions, the amount of phosphate liberated was proportional to the enzyme concentration (cf. Lardy & Wellman, 1953), except with normal mitochondria in the absence of DNP (see Results). The concentration of normal liver mitochondria in the reaction mixture was usually 0·1–0·25 mg. of protein/ml.; most of the experiments with fragments of heart sarcosomes or liver mitochondria were carried out with approx. 0·03–0·1 mg. of protein/ml.

The protein content of the enzyme preparations was determined by the biuret method of Gornall, Bardawill & David (1949), as applied to mitochondrial preparations by Cleland & Slater (1953). The spectrophotometric measurement of the diphosphopyridine nucleotide (DPN) in mitochondria is described by Holton (1955) and by Holton, Hülsmann, Myers & Slater (1957).

Reagents. The crystalline disodium salt of ATP from muscle was supplied by the Sigma Chemical Co., St Louis, Mo., U.S.A. Fresh samples of this reagent appeared to be free of any measurable amounts of inorganic phosphate, adenosine monophosphate (AMP) or ADP. AMP was also obtained from Sigma Chemical Co. ADP was prepared from ATP as described by Slater (1953). Sucrose, KCl and most of the other chemicals were AnalaR reagents supplied by British Drug Houses Ltd., Poole, England. EDTA was also obtained from British Drug Houses Ltd.

Other buffers. The imidazole–HCl buffer was prepared by titrating 0·2M-imidazole with 2N-HCl. The 5:5′-diethylbarbiturate–NaOH buffer was prepared by titrating 0·2M-sodium barbital with 2N-HCl. These were diluted fivefold in the final reaction mixture.

RESULTS

ATPase of isolated liver mitochondria at pH 7·4

The mitochondria isolated from rat-liver homogenates in 0·25M-sucrose had only a small activity towards ATP in the absence of DNP, provided that the isotonic reaction mixture contained EDTA (Table 1). Addition of Mg^{2+} ions to the reaction mixture generally decreased the activity still further (cf. Potter *et al.* 1953; Swanson, 1956). The residual activity towards ATP at pH 7·4 in the presence of Mg^{2+} ions depended on the purity of the ATP. The experiments shown in Table 2 were carried out with an old sample of ATP which contained inorganic phosphate and presumably ADP in amounts equivalent to about 9% of the total molar concentration of ATP; here the amount of free phosphate in the reaction medium actually decreased during the incubation with the liver mitochondria (Table 2). This result, which is similar to that found in some of the experiments reported by Kielley & Kielley (1951), is probably due to the synthesis of ATP from the free phosphate and ADP during the oxidation of endogenous substrates present in the mitochondria. Subsequent experiments were carried out with fresh samples of ATP which did not contain measurable amounts of ADP

Table 1. *Effect of ethylenediaminetetra-acetate on the hydrolysis of adenosine triphosphate by liver mitochondria*

The reaction mixture contained 0·075M-KCl, 0·108M-sucrose and 0·002M-ATP, pH 7·4; various amounts of EDTA, neutralized (pH 7·4) with NaOH, were also added. The mixture was incubated for 15 min. at 20° after the addition of liver mitochondria (0·073 mg. of protein/ml.).

EDTA (M)	μmoles of P/mg. of protein/hr.	
	No DNP	10^{-4}M-DNP
0	4·4	13·2
0·00001	2·7	12·5
0·0001	0·9	11·2
0·001	0·8	10·4
0·01	0·7	10·4

Table 2. *ATPase activity of liver mitochondria after isolation in various media*

Results presented here were obtained in a series of comparative experiments which were carried out during a period of 5 weeks in 1955. Reaction mixture contained 0·075 M-KCl, 0·108 M-sucrose, 0·001 M-EDTA and 0·002 M-ATP, with or without 0·003 M-$MgCl_2$ and with or without 0·0001 M-DNP. The mixture was incubated for 15 min. at 20° and pH 7·4 after addition of 0·2–0·4 mg. of mitochondrial protein/ml. The ATP used for these particular experiments, in contrast with the other experiments reported in this paper, contained appreciable amounts of inorganic phosphate and ADP (see text).

Isolation medium	No. of experiments	Average ATPase activity (μmoles of P/mg. of protein/hr.) No addition	DNP	$MgCl_2$	$MgCl_2$ + DNP
0·25 M-Sucrose	7	0·31	10·9	−0·27	10·0
0·25 M-Sucrose + 0·01 M-tris, pH 7·4	2	−0·13	11·4	+0·13	10·7
0·25 M-Sucrose + 0·01 M-tris, pH 6	1	0·0	—	0·0	8·4
0·25 M-Sucrose + 0·01 M-EDTA, pH 7·4	3	0·77	7·9	2·2	9·4
0·25 M-Sucrose + 0·01 M-EDTA + 0·01 M-$MgCl_2$, pH 7·4	1	0·0	11·8	1·8	11·7

Table 3. *Effect of the concentration of mitochondria on their ATPase activity*

The reaction medium used in the first series of determinations was the same as for Table 2 except that the ATP sample was free of inorganic phosphate and ADP. In the second series of determinations with the same mitochondria, the mixture of 0·075 M-KCl and 0·108 M-sucrose was replaced by 0·233 M-sucrose. The total amount of phosphate liberated by the complete hydrolysis of ATP to ADP should be equal to 2·8 μmoles. The small excess in the presence of Mg^{2+} ions and DNP is probably due to the formation of new ATP by the action of mitochondrial myokinase.

Mitochondria (mg. of protein/ml.)	KCl (M)	μmoles of P/15 min. No addition (*a*)	DNP (*b*)	$MgCl_2$ (*c*)	$MgCl_2$ + DNP (*d*)	(*b*/*a*)	(*d*/*c*)
1·48	0·075	0·15	2·90	0·06	3·55	19·4	59·3
0·74	0·075	0·22	2·78	0·08	2·81	12·6	35·1
0·37	0·075	0·22	2·13	0·08	1·84	9·7	23·0
0·148	0·075	0·14	0·87	0·05	0·74	6·2	14·8
0·074	0·075	0·07	0·44	0·025	0·37	6·3	14·8
1·48	0	0·40	2·42	0·19	2·36	6·0	12·4
0·74	0	0·38	2·13	0·21	1·98	5·6	9·4
0·37	0	0·27	1·48	0·21	1·05	5·5	5·0
0·148	0	0·16	0·55	0·15	0·43	3·4	2·8
0·074	0	0·08	0·28	0·07	0·21	3·5	3·0

Table 4. *Effect of the temperature on the ATPase activity of liver mitochondria*

Incubation temp.	μmoles of P/mg. of protein/hr. $MgCl_2$ (*a*)	$MgCl_2$ + DNP (*b*)	(*b*/*a*)
28°	2·3	28·5	12·4
21	1·3	16·1	12·4
14	0·4	5·8	14·5
1	0·0	0·4	—

or free phosphate. The incubation of the mitochondria with this preparation always resulted in the liberation of a small amount of inorganic phosphate under the same experimental conditions.

The addition of DNP to the reaction mixture increased the activity of the normal mitochondria at pH 7·4 in the presence of Mg^{2+} ions by 10–30 times. Similar increases were reported by Potter & Recknagel (1951), Lardy & Wellman (1953), Robertson & Boyer (1955), Witter, Watson & Cottone (1955) and Swanson (1956). The absolute rates of hydrolysis of ATP in the presence and absence of DNP were also similar to the rates reported by these investigators. As shown by Lardy & Wellman (1953), the presence of KCl was essential for maximal activity in the presence of DNP (Table 3).

The degree of stimulation of the latent ATPase activity was not markedly affected by the temperature of the reaction mixture (Table 4). However, it was highly dependent on the concentration of mitochondria (Table 3). Under our experimental conditions, the amount of phosphate liberated was proportional to the enzyme concentration only when the amount of mitochondrial protein did not exceed 0·5 mg./ml. In the absence of DNP, the amount of phosphate liberated actually decreased when much larger concentrations of mitochondria were used. The specific activity in the absence of DNP (μmoles of P/mg. of protein/hr.) thus decreased sharply from 2·52, with the two lower concentrations of mitochondria, to 0·27, with 1·48 mg. of protein/ml.

The pH of the liver homogenates and mitochondrial suspensions during the normal isolation procedure was in the range 6·5–6·7, regardless of the initial pH of the unbuffered sucrose isolation medium. The ATPase activities, measured under the four conditions in Table 2, were not appreciably affected by the addition of tris buffer to the sucrose solution in order to maintain the pH at 7·4 or at 6·0.

Addition of EDTA to the sucrose solution resulted in mitochondrial preparations with a moderately high ATPase activity in the absence of DNP (Table 2). This may be correlated with the observation that a distinct separation between the mitochondria and the fluffy layer was not obtained after the high-speed centrifuging in the EDTA–sucrose medium and with the fact that the addition of EDTA alters the structure of the microsomal fraction (Palade & Siekevitz, 1956). The ATPase activities could not be restored to normal by subsequently washing the mitochondria with 0·25 M-sucrose.

On the other hand, the EDTA did not seem to exert any deleterious effect on the normal mitochondria after they had been isolated in the usual way in sucrose solution; the mitochondria retained a low activity towards ATP when they were stored for 3–4 hr. at 0° either in sucrose or in sucrose solution containing EDTA. Moreover, normal mitochondria were more stable at 30° when EDTA was added to the suspension (Table 5). In the absence of EDTA, the ATPase activity was increased by pre-incubation for 2 hr. at 30° in sucrose solution (Kielley & Kielley, 1951; Lardy & Wellman, 1953; Potter *et al.* 1953). They also lose the ability to oxidize β-hydroxybutyrate (Table 5); a direct assay showed that the total amount of DPN in the mitochondria was decreased from 4·66 to 0·34 μ-mole/g. of protein, by incubation for 2 hr. at 30°. Both the inactivation of the β-hydroxybutyrate oxidase system and the activation of the ATPase were hastened by the addition of small amounts of Ca^{2+} ions (cf. Potter *et al.* 1953) and delayed by the addition of EDTA (Table 5). Similar results have been observed with heart sarcosomes (Slater & Cleland, 1953).

pH–*activity curves*

The pH–activity curves obtained with normal liver mitochondria in various reaction mixtures are shown in Fig. 1. The effect of EDTA in reducing the activity, at pH 7·4, in the absence of DNP is readily apparent. It had little effect in the presence of DNP. Addition of a small excess of Mg^{2+} ions did not significantly alter the curve obtained in the presence of DNP but decreased the small activity in the absence of DNP to still lower levels. Probably

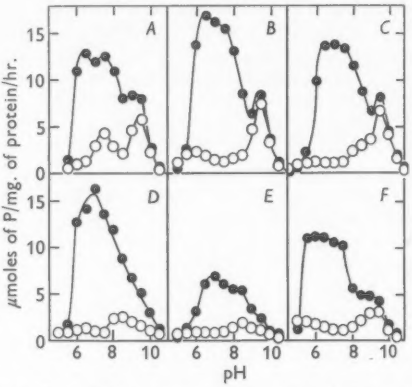

Fig. 1. Rate of hydrolysis of ATP by normal liver mitochondria at various pH values, in the absence (○) and the presence (●) of 10^{-4} M-DNP. The reaction mixture contained 0·075 M-KCl, 0·05 M-sucrose, 0·05 M-tris buffer, 0·002 M-ATP and other components as follows: *A*, one experiment with no further additions; *B*, the average of three experiments with 0·0006 M-EDTA added; *C*, the average of twelve experiments with 0·0006 M-EDTA + 0·001 M-$MgCl_2$; *D*, one experiment with 0·0006 M-EDTA + 0·005 M-$MgCl_2$; *E*, one experiment with 0·0006 M-EDTA + 0·010 M-$MgCl_2$; *F*, one experiment with 0·01 M-EDTA.

Table 5. *Effect of pre-incubation at* 28° *on the enzymic activities of liver mitochondria*

A suspension of normal liver mitochondria in 0·25 M-sucrose with or without added EDTA or $CaCl_2$ was pre-incubated for different periods of time at 28°. Subsequently the ATPase activity was determined in the usual manner and the rate of oxidation of β-hydroxybutyrate was measured manometrically, in the medium of Copenhaver & Lardy (1952) with 0·001 M-EDTA added.

Pre-incubation at 28°		Oxidation of β-hydroxybutyrate	ATPase activity (μmoles of P/mg. of protein/hr.)	
Medium	Time (hr.)	(μl. of O_2/mg. of protein/hr.)	$MgCl_2$	$MgCl_2$ + DNP
0·25 M-Sucrose	0·0	39·5	−0·14	11·7
0·25 M-Sucrose	0·5	20·4	9·0	15·9
0·25 M-Sucrose	2·0	1·1	12·8	—
0·25 M-Sucrose + 0·002 M-EDTA	0·5	34·4	3·6	10·0
0·25 M-Sucrose + 0·002 M-$CaCl_2$	0·5	2·6	10·2	11·0

the presence of Mg^{2+} ions helps to maintain the normal mitochondrial structure during the incubation period of 15 min. at 20° (cf. Perry, 1956; Baltscheffsky, 1956). Higher concentrations of Mg^{2+} ions (0·005 M), approaching the values used in most experiments on oxidative phosphorylation, inhibit the ATPase activity at the higher pH values, and still higher concentrations inhibit the activity over the whole range of pH values (Fig. 1). Low concentrations of Mg^{2+} ions were used in subsequent experiments.

While these experiments were in progress, the effect of the pH on the hydrolysis of ATP by normal liver mitochondria in the absence of DNP was described by Swanson (1956). She also found a low ATPase activity at pH 7·0–7·5 but a much higher ATPase activity both at lower and at higher pH values. Under the conditions used in the present investigations normal liver mitochondria hydrolyse ATP slowly at all pH values between 4·5 and 8·5; a high ATPase activity is observed only in the region of pH 9·4–9·5 (Figs. 1, 2).

A comparison of the effects of different concentrations of DNP showed that 10^{-5} M-DNP produces a partial stimulation of the latent ATPase activity, which is greatest at about pH 6·5 (Fig. 2). A concentration which uncouples oxidative phosphorylation completely (10^{-4} M-DNP) produces no stimulation of the ATPase activity below pH 5, a maximal stimulation at pH 6·5, a partial stimulation in the region of pH 8·5 and no significant stimulation above pH 9·5 (Figs. 1, 2). When the concentration of DNP was increased to 10^{-3} M, the degree of stimulation at pH 6·5 was decreased, whereas at pH 8·5 it was increased (Fig. 2). Apparently, high concentrations of DNP inhibit the ATPase activity of normal mitochondria at pH 6·5; on the other hand, DNP did not exert any marked inhibitory effect on the ATPase activity after the enzymes had been activated by fragmentation of the mitochondria by freezing and thawing (Fig. 3).

Factors affecting the shape of the pH–*activity curves.* The effect of three different buffer systems is illustrated in Fig. 4. The results obtained with tris–HCl and histidine–HCl buffers were practically identical with those obtained with the usual tris

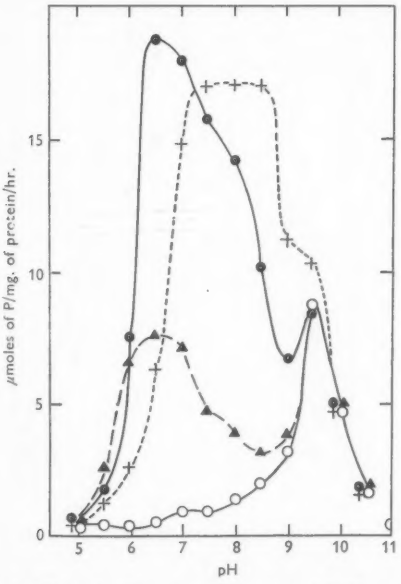

Fig. 2. Effect of different concentrations of DNP on the rate of hydrolysis of ATP by normal liver mitochondria. The reaction mixture contained 0·075 M-KCl, 0·05 M-sucrose, 0·05 M-tris buffer, 0·002 M-ATP, 0·001 M-$MgCl_2$ and 0·0006 M-EDTA. The following concentrations of DNP were added: ○, none; ▲, 10^{-5} M; ●, 10^{-4} M; +, 10^{-3} M.

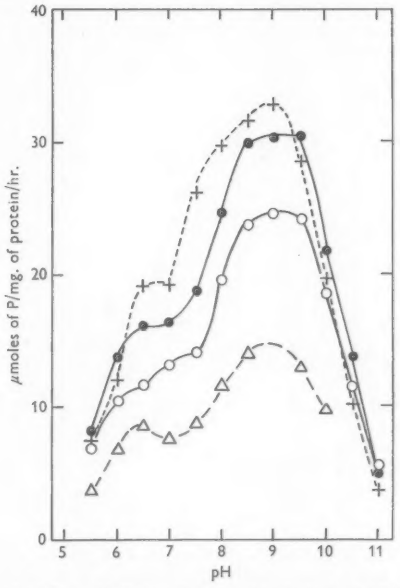

Fig. 3. Effect of DNP on the rate of hydrolysis of ATP by preparations of fragmented liver mitochondria. The reaction mixture was the same as given for Fig. 2. ATPase activities of the mitochondria were measured in the absence of DNP after the mitochondrial preparations had been subjected to freezing and subsequent thawing once (△) and twice (○). Activities of the latter preparation were also measured in the presence of 10^{-4} M-DNP (●) and of 10^{-3} M-DNP (+).

buffer (containing acetic acid and, at the higher pH values, aq. NH_3, see p. 560). 5:5-Diethylbarbiturate–NaOH and imidazole–HCl buffers appeared to be less satisfactory, since the maximum rate of hydrolysis of ATP was decreased appreciably; however, the shape of the pH–activity curves was not altered greatly. A few experiments were also carried out with no buffer apart from the substrate. The ATP had little buffer capacity in the reaction mixture above pH 8; however, the results obtained up to this point were similar to those obtained in the presence of tris buffer. It seems probable therefore that the pH–activity curves are not distorted in any way by the presence of tris, acetate and (at the higher pH) NH_4^+ ions in the reaction mixtures.

The effect of the concentration of Mg^{2+} ions on the ATPase activity of normal mitochondria was mentioned above (cf. Fig. 1). This was studied in more detail with a fully activated preparation of aged mitochondria which had been subjected to repeated freezing and thawing (Fig. 5). Normal mitochondria contain appreciable amounts of bound magnesium (Siekevitz & Potter, 1955) and do not require added Mg^{2+} ions to be able to hydrolyse ATP at a maximal rate in the presence of DNP (cf. Fig. 1) (Lardy & Wellman, 1953; Potter *et al.* 1953). However, after ageing, followed by freezing and thawing, the mitochondrial suspensions have little ATPase activity in the absence of added Mg^{2+} ions (Fig. 5). The pH–activity curve obtained with our preparation of frozen mitochondria in the presence of Mg^{2+} ions is similar to that given by Kielley & Kielley (1953) for their purified preparation of mitochondrial fragments. The effect of the concentration of Mg^{2+} ions is also similar to that reported by Kielley & Kielley (1953), when the differences in substrate concentration are taken into consideration; as shown by Kielley & Kielley (1953), the optimum concentration of Mg^{2+} ions is directly proportional to the concentration of ATP. The maximum activity in our experiments was produced

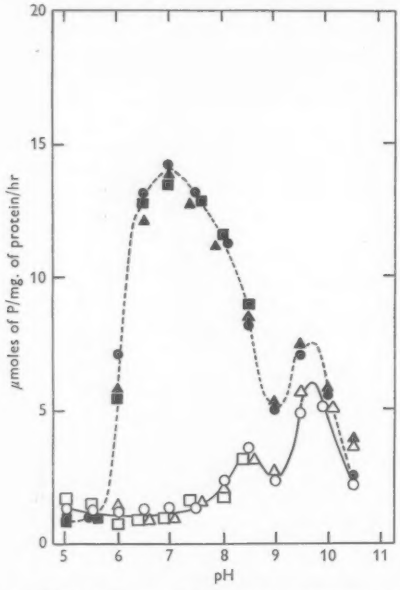

Fig. 4. Effect of different buffers on the hydrolysis of ATP by normal mitochondria. The reaction mixture was the same as that given for Fig. 2 with the exception of the buffer system. The ATPase activities of a normal mitochondrial preparation were measured in the presence of the usual tris buffer (0·05 M), with (●) and without (○) 10^{-4} M-DNP; in the presence of tris–HCl buffer with (▲) and without (△) 10^{-4} M-DNP, and in the presence of histidine–HCl buffer, with (■) and without (□) 10^{-4} M-DNP. For the composition of these buffers see Methods, p. 560.

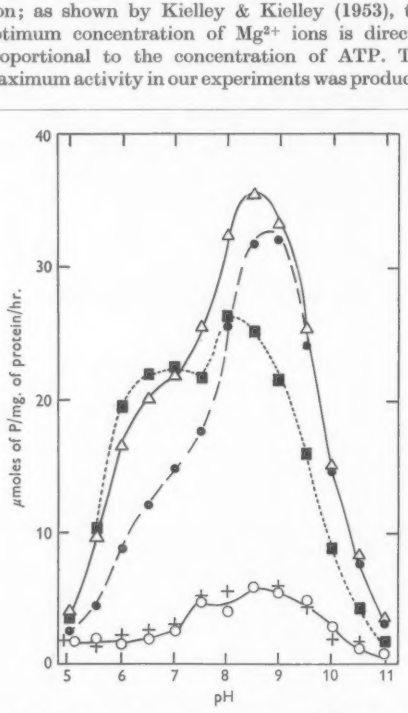

Fig. 5. Effect of different concentrations of $MgCl_2$ on the hydrolysis of ATP by a preparation of liver mitochondria, after ageing, followed by freezing and thawing. The reaction mixture was the same as that given for Fig. 1*A*. Liver mitochondria were aged by incubation for 2 hr. at 30° in 0·25 M-sucrose and subsequently frozen and thawed five times in succession. ATPase activities were measured with no added $MgCl_2$ (○) and with 0·0003 M- (●), 0·001 M- (△) and 0·003 M-$MgCl_2$ (■) added. The effect of 0·001 M-$CaCl_2$ (+) was also determined.

with a concentration of Mg^{2+} ions equal to one-half of the molar concentration of ATP. Concentrations of Mg^{2+} ions equal to 1·5 times the molar concentration of ATP caused inhibition above pH 7·5 (Fig. 5). When the Mg^{2+} concentration equalled five times that of the ATP, the ATPase was inhibited at all pH values between 6 and 10; a similar result was observed with normal mitochondria (Fig. 1). The differences in the optimum concentrations of Mg^{2+} ions at different pH values probably reflect the effect of the pH on the dissociation of ATP and its affinity for Mg^{2+} ions (Martell & Schwarzenbach, 1956; Nanninga, 1956). The reason for the inhibitory effect of excess of Mg^{2+} ions is still obscure but it is possible that ATP itself rather than a magnesium complex of ATP serves as the substrate for an enzyme which is only active when combined with magnesium. The effect of the concentration of Mg^{2+} ions on the ATPase activity of heart sarcosomes (isolated according to the procedure of Cleland & Slater, 1953) and sarcosomal fragments was found to be similar to the effects described above. A concentration of 0·001 M-Mg^{2+} ions in the presence of 0·002 M-ATP seemed most suitable for subsequent experiments.

Calcium ions do not stimulate the hydrolysis of ATP by aged mitochondria at pH 7·4 (Lardy & Wellman, 1953), even though calcium is also firmly bound by ATP (Martell & Schwarzenbach, 1956). None of the ATPase activities between pH 5 and 11 was in fact activated by Ca^{2+} ions (Fig. 5); thus these enzymes differ in this respect from the ATPase of myosin (cf. Engelhardt, 1946). On the other hand, the hydrolysis of ATP was activated by Mn^{2+}, Co^{2+}, Zn^{2+}, Fe^{2+} (Fig. 6) and by Sn^{2+} as well as by Mg^{2+} ions. Similar experiments with sarcosomal fragments have shown that Cd^{2+}, Ni^{2+} and Be^{2+} ions also produce a considerable activation; Al^{3+}, Ba^{2+} and Cu^{2+} ions were ineffective or inhibitory. These experiments were carried out at pH 7·4 in the same way as the experiments shown in Fig. 6; other experiments showed that 3×10^{-4} M-$MnCl_2$ and $SnCl_2$ were equally effective over the whole pH range where the salts remained soluble. Activation of the ATP hydrolysis by Mn^{2+} ions has been reported previously for other enzyme preparations which are activated by Mg^{2+} ions (Kielley & Meyerhof, 1948; Meyerhof & Ohlmeyer, 1952; Sacktor, 1953); apparently a large number of bivalent cations are almost equally effective. There was no

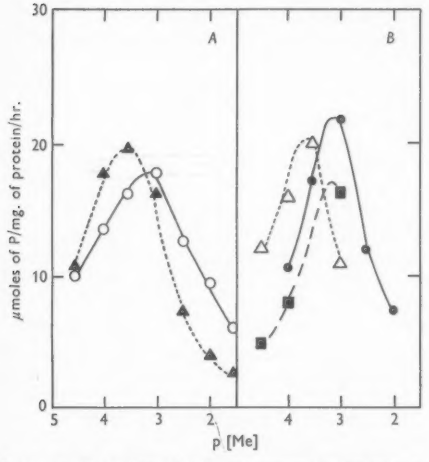

Fig. 6. (*A*, *B*). Effect of different ions on the hydrolysis of ATP by aged liver mitochondria at pH 7·4. The reaction mixture contained 0·075 M-KCl, 0·108 M-sucrose and 0·002 M-ATP, pH 7·4. The liver mitochondria were aged by incubation for 2 hr. at 30° in 0·25 M-sucrose solution and their ATPase activities measured in the presence of various concentrations of $MgCl_2$ (○), $MnSO_4$ (▲), $CoCl_2$ (●), $ZnSO_4$ (△) and $FeSO_4$ (■). ATPase activity is plotted against p[Me], the negative logarithm of the concentration of the ions (g. ions/l.). Similar results were obtained with $ZnSO_4$ and $ZnCl_2$.

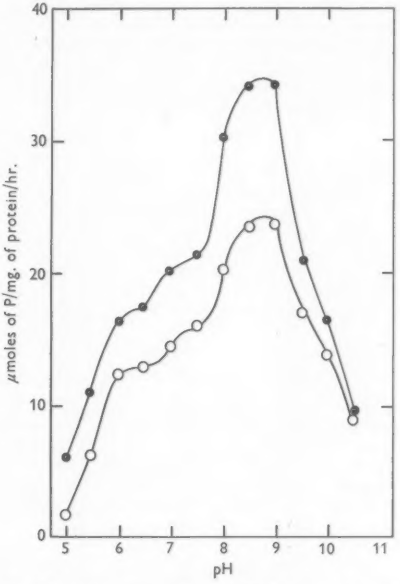

Fig. 7. Effect of the concentration of ATP on the hydrolysis of ATP. Liver mitochondria were aged by incubation for 2 hr. at 30° and subsequently frozen and thawed. ATPase activities were measured in the presence of 0·002 M-ATP + 0·001 M-$MgCl_2$ (○) and of 0·01 M-ATP + 0·005 M-$MgCl_2$ (●) in a reaction medium containing 0·075 M-KCl, 0·05 M-sucrose and 0·05 M-tris buffer.

evidence of a slow reaction between the enzyme and the cation. Identical results were obtained when the mitochondrial preparations were added to a mixture containing Mg^{2+} ions and ATP or when they were pre-incubated with Mg^{2+} ions at 20° for between 15 min. and 3 hr., and the ATP was added later.

The absolute rate of hydrolysis of ATP by frozen mitochondria was increased by 30–50% when the concentration of ATP was increased fivefold (Fig. 7); however, the shape of the pH–activity curve was not altered markedly.

Substrate specificity

Although the enzyme responsible for the hydrolysis of ATP at pH 7·4 appears to be relatively specific for this substrate (Kielley & Kielley, 1953; Lardy & Wellman, 1953; Sacktor, 1953; cf. Perry, 1956), it seemed possible that some of the other optimum pH values might be due to the presence of other enzymes which are not specific for ATP. For example, the normal preparations of liver mitochondria are inevitably contaminated with a small number of lysosomes which contain an acid phosphatase capable of hydrolysing glycerol phosphate (Appelmans, Wattiaux & de Duve, 1955; de Duve, Pressman, Gianetto, Wattiaux & Appelmans, 1955). The presence of this enzyme in our mitochondrial preparations could be confirmed (Table 6); however, the rate of hydrolysis of glycerol phosphate is small in comparison with the rate of hydrolysis of ATP, and was not stimulated by DNP (cf. Appelmans & de Duve, 1955). Similar results were obtained with phenyl phosphate, AMP and inorganic pyrophosphate as substrates (Table 6). It seems improbable that an acid phosphatase, alkaline phosphatase or pyrophosphatase could be responsible for a significant portion of the DNP-stimulated ATPase activity of normal mitochondria.

The hydrolysis of ADP by normal mitochondria was stimulated by the addition of DNP (Table 6), but this can be explained by the combined action of adenylkinase and a DNP-stimulated ATPase.

Table 6. *Hydrolysis of different phosphate esters by normal liver mitochondria*

The reaction medium contained 0·075 M-KCl, 0·05 M-sucrose, 0·05 M-tris buffer, 0·001 M-$MgCl_2$, 0·0006 M-EDTA and 0·002 M-phosphate ester, with or without 10^{-4} M-DNP. The mixture was incubated for 20 min. at 20° after the addition of 0·18 mg. of mitochondrial protein/ml. The liberation of inorganic phosphate was less than 0·05 μmole/mg. of protein/hr. in all cases where the value is omitted. Parallel experiments showed a similar rate of hydrolysis of the inorganic pyrophosphate and other esters by a preparation of aged and frozen mitochondria, although ATP is hydrolysed more rapidly by these preparations (Figs. 3, 5 and 8).

	Rate of enzymic hydrolysis (μmoles of P/mg. of protein/hr.)					
	ATP		ADP		AMP	
pH	$MgCl_2$	$MgCl_2$ + DNP	$MgCl_2$	$MgCl_2$ + DNP	$MgCl_2$	$MgCl_2$ + DNP
5·0	1·2	1·2	0·2	0·1	0·1	0·1
5·5	1·7	2·6	0·4	0·8	0·2	0·2
6·0	1·4	11·2	0·3	1·4	0·4	0·4
6·5	1·2	12·2	0·2	1·3	0·2	0·2
7·0	1·1	12·2	0·1	1·2	0·2	0·2
7·5	1·2	12·1	0·2	1·1	0·2	0·2
8·0	3·0	10·5	0·7	1·1	0·2	0·2
8·5	3·4	7·8	0·9	1·1	0·2	0·1
9·0	5·1	8·8	0·9	1·1	0·2	0·1
9·5	7·1	9·9	0·9	1·1	0·3	0·1
10·0	5·3	5·7	0·6	0·7	0·2	0·1
10·5	2·5	2·5	0·5	0·5	0·1	0·1
	β-Glyceryl phosphate		Phenyl phosphate		Inorganic pyrophosphate	
	$MgCl_2$	$MgCl_2$ + DNP	$MgCl_2$	$MgCl_2$ + DNP	$MgCl_2$	$MgCl_2$ + DNP
5·0	0·1	0·1	0·4	0·4	0·8	0·2
5·5	0·1	0·2	0·3	0·3	0·4	0·1
6·0	0·2	0·2	0·4	0·4	0·2	0·1
6·5	0·1	0·1	0·1	0·1	0·1	—
7·0	—	—	—	—	0·1	0·1
7·5	—	—	—	—	0·3	0·3
8·0	—	—	—	—	0·2	0·1
8·5	—	—	—	—	0·1	—
9·0	0·1	—	—	—	0·4	—
9·5	—	—	—	—	0·8	—
10·0	—	0·0	—	—	0·3	—
10·5	0·0	0·0	—	—	—	—

ATPase activity at pH 9·4

In contrast with the results obtained at lower pH values, the ATPase activity of normal mitochondria at pH 9·4 is relatively high in the absence of DNP and is scarcely affected by the addition of DNP (Figs. 1–3). It was necessary to consider the possibility that the rapid hydrolysis of ATP by normal mitochondria at pH 9·4 might reflect the effect of the alkaline pH on the structure of the mitochondria. The mitochondria suspensions in a series of tubes containing buffers at different pH values were all equally turbid initially; however, the suspensions gradually became translucent in the buffers above pH 9 during the course of the incubation period of 15 min. at 20°, and no change was observed at lower pH values. However, the slow increase in translucency at pH 9·5 was not associated with an increased ATPase activity, since the amount of ATP hydrolysed at pH 9·5 increased linearly with the time of incubation (Table 7). In the same experiment, concentrated suspensions of the mitochondria were also pre-incubated for 15 min. in the same reaction mixtures at pH 6·0, 7·5 and 9·5; each suspension was subsequently diluted in a series of buffers for determination of the ATPase activity in the usual way. If the mitochondria were seriously damaged by the pre-incubation at pH 9·5, it would be expected that the activity at lower pH values in the absence of DNP should also be increased by this period of pre-incubation. The increase in the activity at lower pH values proved to be relatively small (Table 7). Further evidence will be presented below which suggests that the high ATPase activity at pH 9·4 is due to a separate enzyme system (cf. Figs. 8, 9).

Effect of ageing and fragmentation of the mitochondria. The effect of ageing on the mitochondria has been described by various authors (Kielley & Kielley, 1951; Lardy & Wellman, 1953; Potter *et al.* 1953). After pre-incubation of the mitochondria

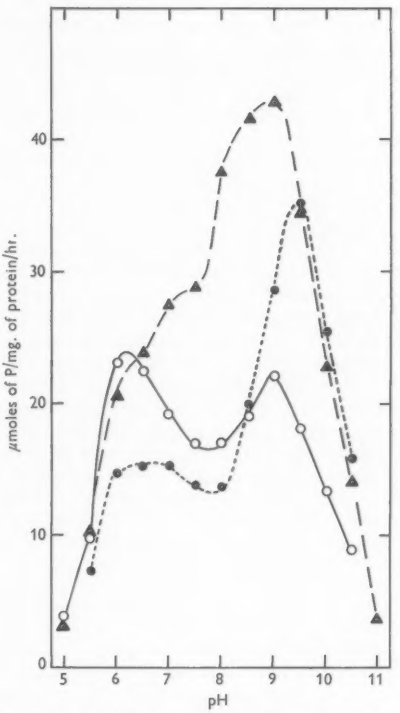

Fig. 8. Rate of hydrolysis of ATP by a preparation of liver mitochondria after subjection to various treatments. The reaction mixture contained 0·075 M-KCl, 0·05 M-sucrose, 0·05 M-tris buffer, 0·002 M-ATP and 0·001 M-$MgCl_2$. A preparation of normal liver mitochondria was aged by incubation for 2 hr. at 30° in 0·25 M-sucrose (○); subsequently a portion of this aged preparation was stored for 1 day at 2° (●), and another portion was stored for 1 day at −18° (▲).

Table 7. *Effect of the incubation period on the latent ATPase activities of normal liver mitochondria*

The reaction medium was the same as for Table 6, with 0·002 M-ATP as substrate and without DNP. In the first series of measurements with no pre-incubation of the mitochondria, the mixtures were incubated at 20° for different periods of time after the addition of 0·25 mg. of mitochondrial protein/ml. In the second series of experiments, a more concentrated suspension of the same preparation of mitochondria (3·75 mg. of protein/ml.) was incubated for 15 min. at 20° in three reaction mixtures of the same composition as was normally used for the ATPase assay; subsequently small samples of these concentrated suspensions were diluted 15-fold in a series of tubes containing the reaction mixtures and the ATPase assay was carried out in the usual manner.

pH during pre-incubation	Incubation period for ATPase assay (min.)	ATPase activity (μmoles of P/mg. of protein/hr.) at pH 5·5–10·0									
		5·5	6·0	6·5	7·0	7·5	8·0	8·5	9·0	9·5	10·0
—	5	1·0	1·3	1·8	1·5	1·6	2·2	2·6	3·2	4·5	3·2
—	15	0·7	0·7	1·0	0·8	1·1	1·7	2·2	2·4	4·8	3·3
—	30	0·7	0·7	1·0	0·8	1·0	1·7	2·2	2·4	4·8	3·2
6·0	15	1·0	0·9	1·3	1·2	1·5	2·8	3·0	3·2	4·5	3·2
7·5	15	1·0	0·8	1·2	1·0	1·4	2·3	2·5	—	4·0	3·2
9·5	15	1·0	1·5	1·9	1·8	2·0	2·6	2·7	3·0	4·5	2·4

suspensions in sucrose solution for 2 hr. at 30°, the rate of hydrolysis of ATP at pH 7·4 in the presence of Mg^{2+} ions reached a value which was consistently a little higher than the maximum observed with normal mitochondria in the presence of DNP (Fig. 8; cf. Potter *et al.* 1953). Considerably higher values were observed at pH 6·3 and 9; the position of the maximum at pH 9 is probably due to the superimposition of two pH–activity curves with maxima near pH 8·5 and 9·4. However, the ATPases are still not fully activated by ageing; the activities at pH 7·4, 8·5 and 9·4 increased greatly when the preparation of aged mitochondria was subsequently frozen and thawed. On the other hand, the ATPase activity at pH 6·3 appeared to have reached its maximum after ageing and was scarcely affected by the freezing procedure. When the suspension of aged mitochondria was stored at 2° for 1 day, the activity at pH 6·3 was considerably reduced, whereas that at pH 9·4 increased to its maximal value. The results obtained in another experiment suggest that similar changes may occur when the aged mitochondria are incubated for a further 3 hr. at 30°. These data show that different degrees of activation of the ATPase activities at different pH values can be achieved by subjecting the mitochondria to various treatments.

The relative heights of the maxima at pH 6·3 and 9 varied considerably from one preparation of aged mitochondria to another. On the other hand, the results obtained with normal mitochondria (Fig. 1) and with the fully activated preparation of aged frozen mitochondria (Fig. 8) were consistently reproducible. The variability in the pH–activity curves obtained with aged mitochondria is probably due to differences in the uncontrolled factors, e.g. the calcium content, pH and concentration of mitochondria in the suspension during the incubation at 30°. In one experiment, two portions of the same suspension of mitochondria were aged separately under aerobic and anaerobic conditions (cf. Hunter, Davis & Corlat, 1956); the results obtained with these two preparations were practically identical.

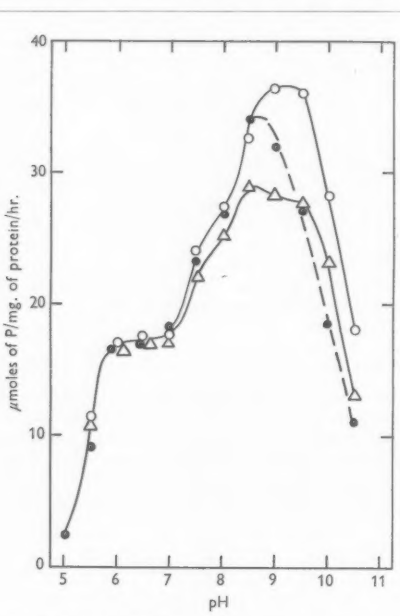

Fig. 9. Hydrolysis of ATP by a preparation of normal liver mitochondria which had been subjected to freezing and thawing three (△), five (○) and six (●) times in succession. The reaction medium is the same as that given for Fig. 8.

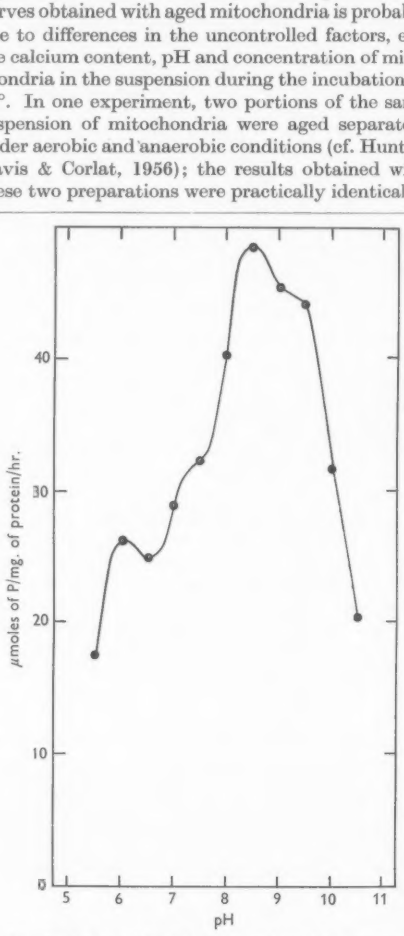

Fig. 10. Hydrolysis of ATP by mitochondrial fragments. A preparation of normal liver mitochondria was suspended in cold 0·003 M-tris buffer, pH 7·5, and homogenized in a Waring Blendor for 10 min. Intact mitochondria and inactivated particles were centrifuged off at 18 000 *g* and the ATPase activities of the mitochondrial fragments in the supernatant fluid were measured in the same reaction medium as given for Fig. 8.

The effect of freezing and thawing is probably due to fragmentation of the mitochondria. A single freezing is sufficient to produce a maximal or near-maximal activation of the ATPase of aged mitochondria. However, the maximal activity was still not attained after freezing and thawing unaged mitochondria five or six times (cf. Figs. 3 and 9). Repeated freezing and thawing of an aged or a normal preparation of mitochondria was sometimes accompanied by a precipitation of some of the protein material and a loss of the ATPase activity above pH 8·5 (Fig. 9).

Activation of the ATPase was also brought about by suspension of normal mitochondria in a hypotonic medium at 0° and treatment in a Waring Blendor (Fig. 10), as described by Kielley & Kielley, 1953 (Fig. 10). However, this preparation was not stable at either −18° or 0° (cf. Hollünger, 1955). The preparations of frozen mitochondria in sucrose solution were also unstable when stored at 0° (Fig. 11), but could be stored in a frozen state at −18° for at least 3 months with little change in activity.

The ATPases present in a fully activated preparation of frozen aged mitochondria were all inactivated to a similar extent when this preparation was allowed to stand for 1 day at 0° in sucrose solutions at various pH values (Fig. 11). The stability of the enzymes appears to be maximal at pH 7–8 under these conditions. A similar result was obtained when the mitochondrial preparations were incubated at more extreme pH values for 30 min. at 20°. The enzymes can also be inactivated by exposure to temperatures above 40° for 30 min. at pH 7·5; again no evidence of a marked selectivity in the degree of inactivation was observed.

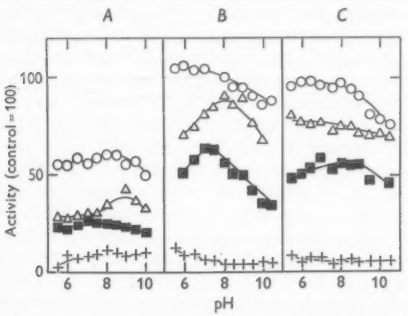

Fig. 11. Stability of the ATPase activities. The reaction medium was the same as that given for Fig. 8. (*A*) ATPase activities of mitochondria which had been frozen and thawed were measured after standing for 23 hr. at 0° in 0·2 M-sucrose + 0·05 M-tris buffer at pH 5 (+), pH 6 (△), pH 7 (○) and pH 9 (■). Similar results were obtained after standing at pH 7 and at pH 8. (*B*) ATPase activities were measured after the mitochondrial preparations were pre-incubated for 30 min. at 20° in 0·05 M-sucrose + tris buffer at pH 3·8 (+), pH 4·8 (△), pH 9·8 (○) and pH 11·5 (■). (*C*) ATPase activities were measured after the mitochondrial preparations were pre-incubated for 30 min. at pH 7·5 in 0·05 M-sucrose + 0·05 M-tris buffer at 35° (○), 45° (△), 50° (■) and 55° (+).

DISCUSSION

The pH–activity curves of the hydrolysis of ATP by liver mitochondria, treated in various ways to activate the latent ATPase, show evidence of four different peaks, at pH 6·3, 7·4, 8·5 and 9·4. The different curves often overlapped one another to give peaks at intermediate pH values, but it was possible under appropriate conditions to demonstrate each peak separately from the others.

The existence of four different peaks could conceivably be explained by one or a combination of the following factors:

(1) The effect of pH on the concentration of the ionic species of ATP (or of an ATP–magnesium complex) which is the substrate of the enzyme.

(2) The effect of pH on the permeability of the mitochondria to ATP.

(3) The presence in mitochondria of four different enzymes (or enzyme systems) which bring about the hydrolysis of ATP.

The first two factors would be expected to be important only at low concentrations of ATP. In our experiments, a high concentration of ATP (0·002 M) in the presence of an optimum concentration of Mg^{2+} ions (0·001 M) was used, and a fivefold increase in these concentrations increased the rate of hydrolysis of ATP only slightly, without altering the shape of the pH–activity curve. Moreover, neither of these factors would explain the differential effects of different concentrations of DNP. For these reasons we consider that the first two factors do not contribute to an important extent to the shape of the pH–activity curves, which we interpret in terms of the presence in mitochondria of four different enzyme systems bringing about the hydrolysis of ATP. These four enzymes will be designated by their optimum pH values, namely 6·3, 7·4, 8·5 and 9·4. This interpretation is strongly supported by our more recent work (Hülsmann & Slater, 1957) which shows that oxidative phosphorylation (as measured by the P:O ratio) in heart sarcosomes has three peaks (at pH 6·3, 7·4 and 8·5) with glutamate as substrate, two with succinate (at pH 7·4 and 8·5) and one with ascorbate (at pH 6·3). Under these conditions the permeability of the mitochondria to ATP cannot be a factor.

The four different enzyme systems are characterized by the following properties.

9·4 *enzyme*. This is the only enzyme which is appreciably active in freshly prepared liver

mitochondria, in the presence of EDTA and in the absence of uncoupling agents (Fig. 1*B*). It was shown that this activity is not due to irreversible structural damage, brought about by the high pH. This enzyme is not stimulated by DNP (up to 10^{-3}M), but is markedly activated by ageing of the mitochondria, followed by freezing and thawing.

8·5 *enzyme*. This enzyme, like the 7·4 and 6·3 enzymes, is stimulated by DNP, but requires a higher concentration of this reagent for full activation. It is most clearly demonstrated by plotting the difference between the activity with 10^{-3}M-DNP and with 10^{-4}M-DNP (Fig. 12). A clear peak at this pH is also shown in mitochondria damaged in the Waring Blendor (Fig. 10), and in the effect of Mg^{2+} ions on the ATPase activity of heart sarcosomes (Holton *et al.* 1957).

7·4 *enzyme*. Although the peak of this enzyme is usually obscured by the presence of neighbouring peaks, an inflexion in this region is often obtained (e.g. Fig. 12). A clear peak was obtained at pH 7·4, when measurements were made in the absence of EDTA, either in the presence or in the absence of DNP or Mg^{2+} ions (Fig. 1*A*; see also Myers & Slater, 1957*a* for another experiment in the presence of 0·001M-$MgCl_2$). This enzyme is also greatly activated by sodium dithionite (Myers & Slater, 1957*b*).

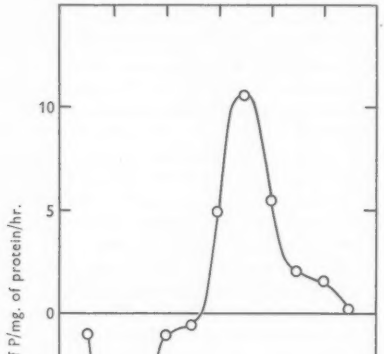

Fig. 12. Difference curve plotting the ATPase activity in the presence of 10^{-3}M-DNP minus the activity in the presence of 10^{-4}M-DNP. The reaction mixture contained 0·075M-KCl, 0·05M-sucrose, 0·05M-tris buffer, 0·002M-ATP and 0·001M-EDTA (no added Mg^{2+} ions). Liver mitochondria, 0·06 mg. of protein/ml.

6·3 *enzyme*. This was the only enzyme activated by 10^{-5}M-DNP (Fig. 2) or by calcium or azide (Myers & Slater, 1957*b*). It is further activated by 10^{-4}M-DNP, but is inhibited by 10^{-3}M-DNP as is clearly seen in Fig. 12. A peak at this pH is also shown in curve 2 of Fig. 1 of Myers & Slater (1957*a*). This enzyme is also activated by incubation for 2 hr. at 30° (Fig. 8) and, unlike the other three enzymes, is not further activated by freezing and thawing the aged mitochondrial preparations.

The decreasing activity of DNP with increasing pH might at first suggest that the activator is the undissociated phenol, which decreases in concentration with increasing pH (cf. Brigham, Brinch-Johnsen & Walaas, 1956). However, this appears extremely unlikely since the pK values of DNP and *p*-nitrophenol are 4 and 7 respectively (Pauling, 1940), so that the concentration of the undissociated phenols in 10^{-4}M-solutions at pH 7·4 are 3×10^{-8}M and 3×10^{-5}M respectively. Nevertheless, *p*-nitrophenol and DNP are about equally effective in uncoupling oxidative phosphorylation. Thus it appears clear that the active agent in DNP is the ion and that changes of pH within the range studied affect the enzyme system rather than the inhibitor.

Liver mitochondria, activated by ageing followed by freezing and thawing, give a pH–activity curve which can also be interpreted in terms of the four enzymes. This curve is dominated by the enzymes in the alkaline region, and the main peak at about 8·8 can be considered to be a composite curve of the 8·5 and 9·4 enzymes. The 6·3 and 7·4 enzymes cause inflexions of the curve (Fig. 7).

Although the four different ATPases were activated to different extents by various means, their stabilities in the fully activated preparation to the treatments described in Fig. 11 were about the same. This could be because a factor common to all four enzyme systems is destroyed by these treatments. The marked inhibition by higher concentrations of Mg^{2+} ions at the higher pH values could be due to the effect of pH on the formation of an inactive magnesium–ATP complex, and does not necessarily represent a different degree of susceptibility of the various enzymes to Mg^{2+} ions.

The results of this investigation confirm earlier suggestions that the hydrolysis of ATP by mitochondrial preparations is not a simple process. The evidence that there are three DNP-sensitive ATPase systems is consistent with the postulate (Hunter, 1951; Lardy & Wellman, 1953) that these enzymes represent a reversal and diversion of the reactions which are responsible for the synthesis of ATP by

oxidative phosphorylation, and with the experimental evidence that there are three DNP-sensitive steps in oxidative phosphorylation. This postulate is given further strong support by our recent finding that oxidative phosphorylation has optimum P:O ratios at the same pH values as the DNP-stimulated ATPase and that the number of optimum values with different substrates is the same as the number of phosphorylating steps with these substrates (Hülsmann & Slater, 1957).

SUMMARY

1. Normal liver mitochondria hydrolyse adenosine triphosphate (ATP) slowly at all pH values between 4·5 and 8·5. The addition of an uncoupling agent such as 2:4-dinitrophenol produces a marked stimulation of the ATPase activity between pH 6 and 8·5. However, the maximal enzymic activity is only attained when the mitochondrial structure has been disintegrated, e.g. by repeated freezing and thawing.

2. The pH–activity curves obtained with mitochondrial fragments or with normal mitochondria in the presence of 2:4-dinitrophenol appear to represent four superimposed optimum pH values which are located in the regions of pH 6·3, 7·4, 8·5 and 9·4. This is interpreted in terms of the presence in mitochondria of four different ATPase systems. All of these hydrolytic enzymes seem to be relatively specific for ATP.

3. The 9·4 enzyme is the only one which is appreciably active in freshly prepared liver mitochondria, in the presence of ethylenediaminetetra-acetate and absence of uncoupling agents. This activity is not due to irreversible structural damage, brought about by the high pH. This enzyme is not stimulated by 2:4-dinitrophenol but is activated by ageing followed by freezing and thawing.

4. The 8·5 enzyme is stimulated by 2:4-dinitrophenol but requires a higher concentration than the 7·4 and 6·3 enzymes for full activation. It is also activated by damaging the mitochondria.

5. The 7·4 enzyme is stimulated by 2:4-dinitrophenol, by mechanical damage to the mitochondria and by removal of ethylenediaminetetra-acetate from the reaction medium.

6. The 6·3 enzyme is the only one clearly activated by 10^{-5} M-2:4-dinitrophenol, and is also fully activated by ageing the mitochondria.

7. The 6·3, 7·4 and 8·5 enzymes, which are stimulated by 2:4-dinitrophenol, may be related to the three steps in the respiratory chain which are coupled with phosphorylation.

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The Enzymic Hydrolysis of Adenosine Triphosphate by Liver Mitochondria

2. EFFECT OF INHIBITORS AND ADDED COFACTORS

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In the previous paper (Myers & Slater, 1957*a*), experiments were reported which were interpreted on the basis that four different enzymes or enzyme systems were responsible for the hydrolysis of adenosine triphosphate (ATP) by liver mitochondria, under various conditions. Each of these systems appeared to be relatively specific for ATP. Three of the four enzyme systems were activated by dinitrophenol (DNP), which suggested that they contained components which might be involved in oxidative phosphorylation.

In the present paper, the effect of various inhibitors of the respiratory chain on the hydrolysis of ATP is examined. This investigation was undertaken in order to determine whether any of the members of the respiratory chain are directly involved in the enzymic hydrolysis of ATP by mitochondrial preparations, as has been suggested in some formulations of oxidative phosphorylation (e.g. Slater, 1953*a*).

A preliminary report of some of these results has appeared (Myers & Slater, 1957*b*).

METHODS

All the methods have been described in the previous paper (Myers & Slater, 1957*a*).

The diphosphopyridine nucleotide (DPN) was obtained from the Sigma Chemical Co., St Louis, Mo., U.S.A., and the reduced diphosphopyridine nucleotide (DPNH) was prepared by reduction with alcohol dehydrogenase, as described by Slater (1953*b*).

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RESULTS

Cyanide. Chance & Williams (1956) have shown that freshly prepared liver mitochondria contain endogenous substrate, which is rapidly exhausted by the addition of phosphate acceptor or an uncoupling agent such as DNP in the presence of oxygen. One would expect that under the conditions of the measurement of the ATPase activity in this paper, the endogenous substrate would be rapidly exhausted and that all the components of the respiratory chain would be completely oxidized for the greater part of the assay. So far as the cytochrome system was concerned, this was confirmed by direct spectroscopic observation. The addition of 10^{-3} M-KCN prevented the oxidation of endogenous substrate, and maintained the cytochromes (and presumably also the pyridine nucleotides and flavoproteins) in the reduced state during the whole course of the assay. However, Fig. 1*A* shows that this addition had no effect on the ATPase activity throughout the range of pH values studied.

Similarly, the addition to the Keilin & Hartree (1947) heart-muscle preparation of 10^{-3} M-KCN, either alone or in the presence of 0·01 M-succinate (added to reduce the respiratory chain, since this preparation is free from endogenous substrate), had no effect on the ATPase activity. It must be concluded therefore that neither cytochrome a_3, which combines with cyanide, nor the other members of the respiratory chain which undergo oxidation and reduction under these conditions are likely to be involved in the hydrolysis of ATP by mitochondrial preparations.

Inhibitors of succinic dehydrogenase. The participation of succinic dehydrogenase in the hydrolysis of ATP by the Keilin & Hartree heart-muscle preparation was excluded by incubation with 0·02 M-KCN for 3 hr. at 38° and pH 7·4. Under these conditions, succinic dehydrogenase is completely inactivated (Tsou, 1951). Nevertheless, there was no effect on the rate of hydrolysis of ATP at any pH value between 5 and 10. Further evidence that the succinic dehydrogenase is not involved in this reaction was afforded by lack of effect of 0·01 M-malonate on the ATPase activities of preparations of liver mitochondria.

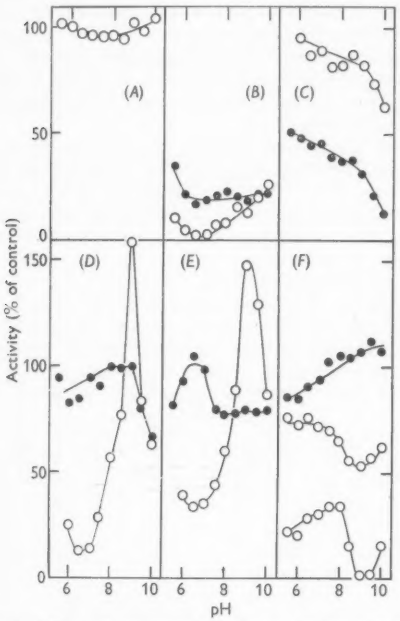

Fig. 1. Effect of various inhibitors on the hydrolysis of ATP by liver mitochondria. Data are presented for the ATPase activities of normal mitochondria in the presence of 10^{-4} M-DNP (○) and of aged and frozen mitochondria (Myers & Slater, 1957*a*) (●); a significant stimulation of the ATP hydrolysis by normal mitochondria in the presence of 10^{-4} M-DNP is produced only between pH 6 and 8·5 (see Myers & Slater, 1957*a*). The following inhibitors were used: (*A*) 10^{-3} M-KCN; (*B*) 10^{-3} M-NaN_3; (*C*) 0·01 M-guanidine hydrochloride; (*D*) 10^{-4} M-*p*-chloromercuribenzoate; (*E*) 0·06 M-KF; (*F*) bovine plasma albumin in concentrations of 5, 3·3 and 10 mg./ml. from top to bottom. The reaction mixture with the normal mitochondria was: KCl (0·075 M); sucrose (0·05 M); tris buffer (0·05 M); $MgCl_2$ (0·001 M); ethylenediaminetetra-acetic acid (6×10^{-4} M); ATP (0·002 M). With aged mitochondria the ethylenediaminetetra-acetic acid was omitted.

Antimycin and 2:3-dimercaptopropanol. Both of these compounds can interrupt the respiratory chain at some point between cytochrome *b* and cytochrome *c* (Slater, 1949; Potter & Reif, 1952). A concentration of antimycin sufficient to stop respiration (10^{-6} M) had no significant effect on the hydrolysis of ATP by normal mitochondria in the presence of DNP or by aged and frozen mitochondria between pH 5 and 10. Higher concentrations of antimycin stimulated the hydrolysis of ATP by normal mitochondria in the absence of DNP. Incubation of the Keilin & Hartree heart-muscle preparation with 0·01 M-2:3-dimercaptopropanol for 30 min. at 37°, a procedure sufficient to inactivate the respiratory chain completely (Slater, 1949), caused only a slight inactivation of the ATPase at all pH values.

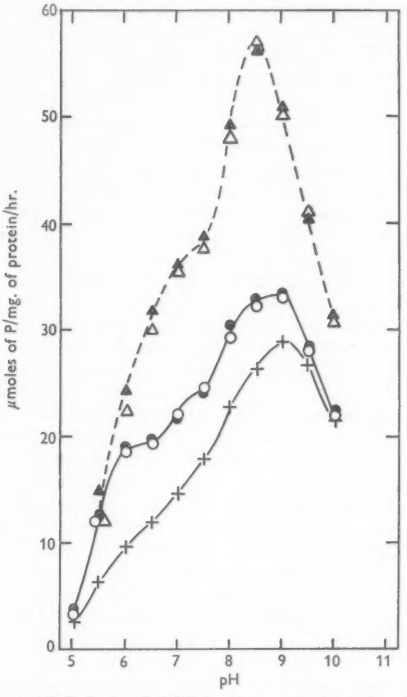

Fig. 2. Hydrolysis of ATP by liver mitochondria after extraction of the cytochrome *c*, as described by Schneider *et al.* (1948). Data are given for the extracted mitochondria in the absence of DNP (+), in the presence of 10^{-4} M-DNP (○) and in the presence of 10^{-4} M-DNP + 10^{-4} M-cytochrome *c* (●). The same mitochondria were also assayed in the absence (△) and the presence (▲) of 10^{-4} M-cytochrome *c* after the mitochondrial preparation had been aged and frozen. The reaction mixtures were the same as in Fig. 1.

Addition and removal of cytochrome c. The hydrolysis of ATP by mitochondria in the presence or absence of DNP was scarcely affected by the addition of 7×10^{-5} M-cytochrome *c*, even after the mitochondria were washed twice with water at 0° and subsequently twice with 0·15 M-NaCl at 0° in order to extract the cytochrome *c* (Schneider, Claude & Hogeboom, 1948). Although the exposure to hypotonic conditions activated the latent ATPase activity to a considerable extent, the addition of DNP still stimulated the ATPase activity (Fig. 2). After this mitochondrial preparation was aged and frozen (Myers & Slater, 1957*a*), the pH–activity curve of the fully activated ATPases appeared to be the same as obtained with a similar preparation from normal mitochondria [cf. Fig. 2 with Fig. 8 of the previous paper (Myers & Slater, 1957*a*)]. The curve was not affected by the addition of 10^{-4} M-cytochrome *c*, either alone or in the presence of dithionite ($Na_2S_2O_4$) in order to reduce the cytochrome *c*. The effect of dithionite itself on the ATPase activity will be described below.

Removal and addition of diphosphopyridine nucleotide and reduced diphosphopyridine nucleotide. Both aged liver mitochondria and the Keilin & Hartree heart-muscle preparation, which contain little DPN, exhibit a high ATPase activity, which was not affected by the addition of 10^{-4} M-DPN or DPNH. The same concentration of DPNH also had no effect on the ATPase activity of fresh liver mitochondria in the presence of DNP, between pH 5·5 and 8·5.

α-Tocopherol and vitamin K_1. There are some indications that these two vitamins may also be involved in oxidative phosphorylation, either as members of the respiratory chain or as essential cofactors (Martius, 1956; Nason & Lehmann, 1956; Bouman & Slater, 1956). The Keilin & Hartree heart-muscle preparation contains an appreciable amount of tocopherol, but no vitamin K (Bouman & Slater, 1956). The ATPase activities of the Keilin & Hartree heart-muscle preparation were not affected by the addition of 0·07 mg. of DL-α-tocopherol or 0·02 mg. of vitamin K_1/ml. of reaction mixture; the stable stock suspensions used in these experiments were prepared in sucrose solutions containing 10 % of ethanol and 0·5 % of plasma albumin (cf. Nason & Lehmann, 1956). The same amount of tocopherol also had no effect on the ATPase activities of normal liver mitochondria in the presence of DNP.

Azide. One of the most potent inhibitors of the ATPase activities of mitochondrial preparations is sodium azide (Meyerhof & Ohlmeyer, 1952; Novikoff, Hecht, Podber & Ryan, 1952; Sacktor, 1953; Robertson & Boyer, 1955; Maruyama, 1954, 1956). However, its action is rather complicated since it also uncouples oxidative phosphorylation (Loomis & Lipmann, 1949) and can stimulate as well as inhibit the ATPase activity of normal liver mitochondria (Robertson & Boyer, 1955; Swanson, 1956). Our experiments showed that the stimulation of the ATPase activity by azide in the absence of DNP precedes the slower inhibitory reaction; the major portion of the ATP hydrolysis occurred within the first 5 min. after adding the normal liver mitochondria to a reaction mixture containing 10^{-3} M-NaN_3. The slow rate of the inhibitory reaction could also be demonstrated by experiments in which the mitochondria were incubated with azide before the ATP was added (Fig. 3); the stimulation by the azide became much less after pre-incubation for 10 min. and disappeared completely when the mitochondria were pre-incubated for 20 min. The stimulation of the latent ATPase activity of normal mitochondria by 10^{-3} M-NaN_3 was maximal at about pH 6 (Fig. 4); this result is similar to that observed with low concentrations of DNP (Myers & Slater, 1957*a*).

The same concentration of azide produced a strong inhibition of the ATPase, activated by the addition of DNP to normal mitochondria or by ageing and freezing the mitochondrial suspensions (Fig. 1*B*). A similar inhibition was observed with heart sarcosomes and with the Keilin & Hartree heart-muscle preparation.

Calcium. Low concentrations of Ca^{2+} ions cause a reversible uncoupling of oxidative phosphorylation, and higher concentrations result in a number of irreversible effects (Potter, Siekevitz & Simonson, 1953; Chance & Williams, 1956). As would be

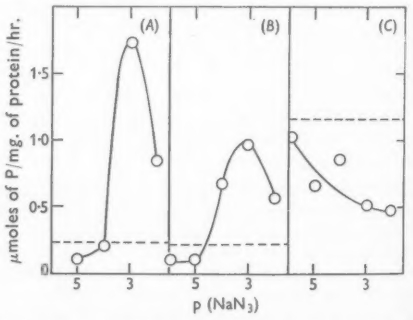

Fig. 3. Effect of sodium azide on the hydrolysis of ATP by normal liver mitochondria. The initial reaction mixture contained 0·075 M-KCl, 0·108 M-sucrose, 0·001 M-ethylenediaminetetra-acetic acid, 0·003 M-$MgCl_2$, pH 7·4. p (NaN_3) indicates the negative logarithm of the molar concentration of NaN_3 added. The mitochondria were pre-incubated in these reaction mixtures with or without azide for (*A*) 0, (*B*) 10 and (*C*) 20 min. before adding a small volume of ATP solution (final concn.: 0·002 M-ATP). The control activities in the absence of azide are indicated by the horizontal broken lines.

expected, low concentrations of Ca^{2+} ions (10^{-3} M) also stimulated the latent ATPase activity of normal mitochondria (cf. Lardy & Wellman, 1953; Potter *et al.* 1953), which was maximal at about pH 6·5 (Fig. 4). The same concentration inhibited the ATPase activity of normal mitochondria at high pH values, and a similar inhibition above pH 9 was also observed with mitochondria, fully activated by ageing followed by freezing and thawing. This inhibition at high pH, which was also obtained with Mg^{2+} ions (Myers & Slater, 1957*a*), is probably due to combination with the ATP (Martell & Schwarzenbach, 1956).

p-Chloromercuribenzoate. This compound is a potent inhibitor of the mitochondrial ATPase at pH 7·4 (Novikoff *et al.* 1952; Sacktor, 1953; Lardy & Wellman, 1953; Hunter, 1956) but, like azide, can also activate the latent ATPase activity of normal mitochondria (Hunter, 1956). However, the activation occurred at all pH values from 5·5 to 9 (Fig. 4) and was not restricted to the lower pH values as was found with low concentrations of DNP, azide and Ca^{2+} ions. Alterations in the translucency of the normal mitochondrial suspensions were also observed (cf. Hunter, Davis & Corlat, 1956). The stimulation of the latent ATPase activity by *p*-chloromercuribenzoate may therefore be due to a general damage of the mitochondrial structure rather than to a specific uncoupling action.

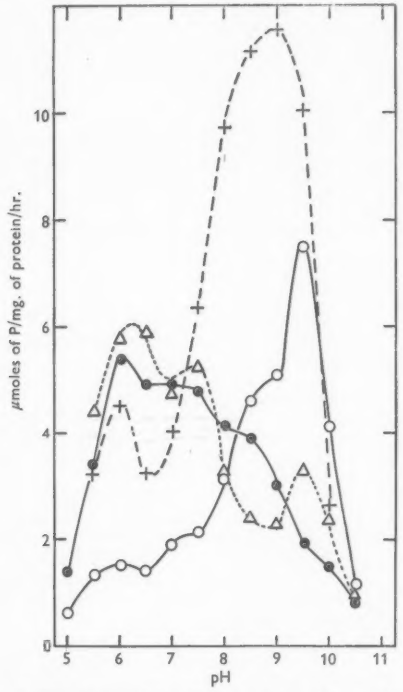

Fig. 4. Stimulation of the ATPase activities of normal liver mitochondria by various compounds. The reaction mixture contained 0·075 M-KCl, 0·05 M-sucrose, 0·05 M-tris buffer, 0·002 M-ATP, 0·001 M-$MgCl_2$ and 0·0006 M-ethylenediaminetetra-acetic acid. The ATPase activities were measured in the presence of 10^{-3} M-$CaCl_2$ (△), 10^{-3} M-NaN_3 (●) and 10^{-4} M-*p*-chloromercuribenzoate (+), the enzymic reaction being allowed to proceed for 15 min. in the first case and for 5 min. in the second and third cases. The control activities in the absence of these compounds were similar in these experiments and the average values obtained are shown above (○).

The degree of inhibition of the activated ATPase by *p*-chloromercuribenzoate was highly dependent on the Mg^{2+} ions and salt concentrations in the reaction medium and on the state of the mitochondria (Lardy & Wellman, 1953). The ATPase of normal mitochondria in the presence of DNP was strongly inhibited by 10^{-4} M-*p*-chloromercuribenzoate between pH 6 and 8, but a stimulation was observed in the region of pH 9 (Fig. 1*D*), where DNP has little effect on the latent ATPase activity but where the *p*-chloromercuribenzoate itself also stimulated in the absence of DNP (cf. Fig. 4). The same concentration of *p*-chloromercuribenzoate had little effect on the hydrolysis of ATP by mitochondria activated by ageing followed by freezing and thawing (Fig. 1*D*). A higher concentration (10^{-3} M) also had little effect. This might suggest that the hydrolysis of ATP by normal mitochondria in the presence of DNP follows a different pathway from the hydrolysis by aged and frozen mitochondria; however, the lack of inhibition by *p*-chloromercuribenzoate in the latter case could be due to the liberation of sulphydryl groups during the process of ageing, and a consequent protection of the ATPases against the inhibitory action. The ATPase activities of the Keilin & Hartree heart-muscle preparation were inhibited 40–60% by 10^{-3} M-*p*-chloromercuribenzoate.

Fluoride. This compound is a strong inhibitor of the pyrophosphatase and adenylkinase (Barkulis & Lehninger, 1951; Swanson, 1952; Sacktor, 1953; Siekevitz & Potter, 1953), but has less effect on the ATPase activity of liver mitochondria at pH 7·4 (Kielley & Kielley, 1951; Novikoff *et al.* 1952; Sacktor, 1953; Lardy & Wellman, 1953; Potter *et al.* 1953). However, the hydrolysis of ATP by normal mitochondria in the presence of DNP could be inhibited by high concentrations of KF (0·06 M) between pH 6 and 8; as with *p*-chloromercuribenzoate, an activation was observed at higher pH values (Fig. 1*E*). On the other hand, 0·06 M-KF had little effect on the hydrolysis of ATP by aged and frozen mitochondria (Fig. 1*E*), aged mitochondria or the Keilin & Hartree heart-muscle preparation.

We did not observe either an uncoupling of oxidative phosphorylation (cf. Middlebrook & Szent-Györgyi, 1955) or a stimulation of the latent ATPase activity of normal mitochondria by high concentrations of KBr and KI. On the other hand, high concentrations of KF did exert some uncoupling action (cf. Kielley & Kielley, 1951) and also stimulated the latent ATPase activity of normal mitochondria in the absence of DNP. However, an appreciable stimulation was observed only in the absence of Mg^{2+} ions, possibly because the mitochondria are then more fragile. The concentration of fluoride (about 0·01 M) used by Copenhaver & Lardy (1952) and most other investigators in experiments on oxidative phosphorylation, had no significant effect on the latent ATPase activity of normal mitochondria in the presence of 0·001 M-$MgCl_2$ and 0·0006 M-ethylenediaminetetra-acetic acid.

Guanidine. Hollünger (1955) has shown that guanidine inhibits the hydrolysis of ATP by aged mitochondria at pH 7·4 but has little effect on the hydrolysis by normal mitochondria in the presence of DNP. These observations were confirmed over the whole pH range in the present investigation, with 0·01 M-guanidine (Fig. 1*C*). These results might also indicate different pathways for the ATPase activities in normal and aged preparations. However, the ATPase activity of normal mitochondria in the presence of DNP can be inhibited by higher concentrations of guanidine.

Albumin. The effect of plasma albumin was of particular interest because it is reported to combine with mitochrome, a haemoprotein which is liberated from aged mitochondria, and it uncouples oxidative phosphorylation and stimulates the latent ATPase activities of normal mitochondria in the same way as DNP; both effects are prevented by the addition of serum albumin (Shmukler & Polis, 1953; Polis & Shmukler, 1954).

No evidence could be obtained to suggest that mitochrome played a role in the activity of the ATPase of our aged and frozen suspensions of liver mitochondria. Bovine plasma albumin (fraction V from Armour Laboratories) in concentrations up to 10 mg./ml. (see Fig. 1) and ovalbumin (up to 50 mg./ml.) had no effect. Mitochondrial fragments obtained in such a way as to extract soluble proteins, by the procedure of Shmukler & Polis (1953), still possessed a high ATPase activity, which was not increased by adding the 'soluble' fraction (see Table 1). The high activity of the Keilin & Hartree preparation and of the mitochondrial fragments of Kielley & Kielley (1953) also speak against an easily soluble enzyme being concerned in these activities, since soluble enzymes are removed from these preparations.

Albumin had no effect on the stimulation of the ATPase by azide, but markedly decreased the activity in the presence of DNP (Fig. 1*F*). However, the significance of this apparent inhibition is uncertain since the inhibition was maximum at pH 9·4, where DNP no longer stimulates. Albumin does not prevent the uncoupling action of DNP (Slater & Lewis, 1954).

Dithionite. Dithionite caused a marked activation of the ATPase activity of aged and frozen mitochondria between pH 6 and 9 (Fig. 5). The degree of stimulation by 10^{-3} M-$Na_2S_2O_4$ was maximum at pH 7·5, and negligible above 9; it was not increased when the concentration of dithionite was increased to 10^{-2} M. The activation by 10^{-3} M-$Na_2S_2O_4$ was not affected by the addition of catalase, indicating that it was not caused by H_2O_2 produced by the auto-oxidation of $Na_2S_2O_4$. There was no stimulation of the ATPase activity of normal mitochondria in the presence of 10^{-4} M-DNP; however, this experiment is not conclusive since the $Na_2S_2O_4$ altered the colour of the DNP from yellow to orange.

Other reducing agents did not give the same effect as $Na_2S_2O_4$. Thus 10^{-3} M-$K_4Fe(CN)_6$ brought about only a slight activation, and 10^{-3} M-glutathione (cf. Novikoff *et al.* 1952) and 10^{-3} M-ascorbic acid were slightly inhibitory (Fig. 5). An oxidizing agent, 10^{-3} M-$K_3Fe(CN)_6$, also had no effect on the ATPase activities of aged and frozen mitochondria.

Table 1. *Enzymic hydrolysis of adenosine triphosphate by the soluble and insoluble fractions of aged liver mitochondria*

Normal liver mitochondria were suspended in 0·15 M-KCl + 0·02 M-$NaHCO_3$ and aged by incubation for 80 min. at 30°. The 'soluble' fraction was recovered by centrifuging off the mitochondria at 27 000 *g*. The insoluble mitochondrial material was washed with the salt solution, resuspended and incubated for a further 80 min. at 30° to complete the extraction of soluble proteins. The mixture was again centrifuged at 27 000 *g*, and the sediment washed and resuspended. This constituted the insoluble fraction. The reaction mixture for the ATPase assay contained 0·075 M-KCl, 0·05 M-sucrose, 0·05 M-tris buffer, 0·002 M-ATP and 0·001 M-$MgCl_2$.

	ATPase activity (μmoles of P/mg. of protein/hr.)		ATPase activity of the soluble and insoluble fractions combined (calc. as % of the sum of their separate activities)
pH	Insoluble fraction	'Soluble' fraction	
5·5	10·7	2·9	94
6·0	15·9	2·6	93
6·5	18·7	2·6	95
7·0	22·3	2·8	92
7·5	25·2	3·0	90
8·0	30·1	3·7	86
8·5	34·0	4·1	81
9·0	32·1	3·7	83
9·5	27·8	2·7	95
10·0	22·0	1·7	96

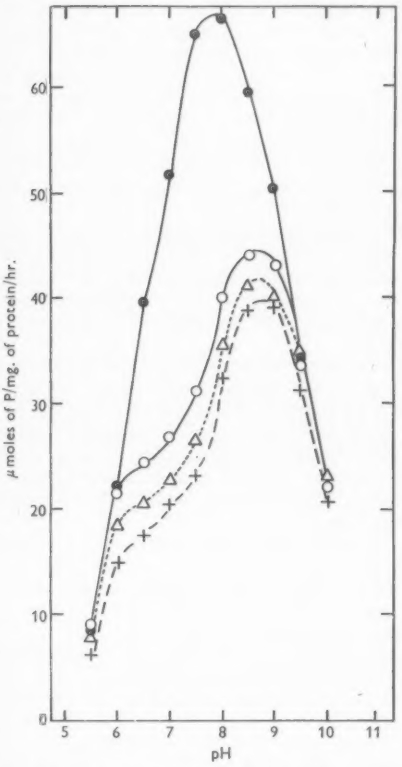

Fig. 5. Effect of reducing agents on the ATPase activities of aged and frozen liver mitochondria. The reaction mixture is the same as that given in Table 1. ATPase activities were measured in the absence of further addition (○) and in the presence of 10^{-3}M-sodium dithionite (●), 10^{-3}M-glutathione (△) and 10^{-3}M-ascorbic acid (+).

DISCUSSION

The coupling of phosphorylation with the transfer of hydrogen (or electrons) through the respiratory chain has been formulated in its simplest terms by the reactions (1) and (2):

$$AH_2 + B + C \rightleftharpoons A \sim C + BH_2 \quad (1)$$

$$A \sim C + ADP + H_3PO_4 \rightleftharpoons A + C + ATP \quad (2)$$

where AH_2 and B are adjacent members of the respiratory chain, whose interaction is coupled with phosphorylation, and C is an intermediate linking the oxidation–reduction with the phosphorylation reaction (Slater, 1953*a*). It was postulated that DNP was in some way able to bring about the hydrolysis of A~C [reaction (3)]:

$$A \sim C + H_2O \xrightarrow{(DNP)} A + C. \quad (3)$$

The hydrolysis of ATP stimulated by DNP is brought about by the reverse of reaction (2), followed by reaction (3).

According to this formulation, a member of the respiratory chain (A), as well as a component (C), which does not undergo oxidation–reduction, is directly involved in the hydrolysis of ATP. Therefore combination of A with an inhibitor or its reduction to AH_2 would be expected to inhibit the ATPase activity. In this paper it has been shown that the addition of inhibitors of the respiratory chain (cyanide, antimycin, malonate), or the removal or inactivation of certain components (DPN, cytochrome *c*, the 2:3-dimercaptopropanol-labile factor), or the reduction of all components of the respiratory chain by the addition of cyanide and substrate, did not cause any inhibition of the ATPase activity. Thus the minimum hypothesis represented by equations (1), (2) and (3) is no longer adequate, and must be modified to take A out of the 'ATPase' reaction. The simplest extension of the theory is to introduce an additional step, with an additional component, between reactions (1) and (2). Chance & Williams (1956) have also given kinetic reasons for introducing an additional component (X) between reactions (1) and (2). They have also pointed out that C in the above scheme has the properties of an inhibitor, since A~C is an inhibited form of A which cannot be reduced in the respiratory chain to AH_2. For this reason, Chance & Williams have replaced C by I (inhibitor). Since it is clearly desirable to avoid a multiplicity of symbols, we shall adopt the symbols X and I of Chance & Williams. We shall not, however, replace AH_2 and B by specific members of the respiratory chain, because we do not think that these have been definitely identified. The modified hypothesis then becomes:

$$AH_2 + B + I \rightleftharpoons A \sim I + BH_2 \quad (1)$$

$$A \sim I + X \rightleftharpoons X \sim I + A \quad (2)$$

$$X \sim I + ADP + H_3PO_4 \rightleftharpoons X + I + ATP \quad (3)$$

$$X \sim I + H_2O \xrightarrow{(DNP)} X + I \quad (4)$$

The hydrolysis of ATP is now explained by the reverse of reaction (3), followed by reaction (4). The latter reaction might involve DNP–I as an intermediate (Chance & Williams, 1956).

It is probable that there are three oxidation–reduction steps in the respiratory chain which are coupled with phosphorylation. It seems likely that each of the oxidation–reduction pairs (AH_2 and B)

involved would require a different mediator I. Thus there would be three different inhibitors which could bring about the hydrolysis of ATP in the presence of DNP by the reversal of reaction (3), followed by reaction (4). It is possible that the three different DNP-sensitive enzyme systems suggested in the previous paper (Myers & Slater, 1957*a*) are reactions involving the three different inhibitors. In the absence of evidence to the contrary, it is simplest to assume that X is the same in all cases and could, in fact, represent a general enzyme or coenzyme of respiratory-chain phosphorylation.

Assuming that the three peaks obtained under different conditions with DNP represent three different enzymes, all three are inhibited by azide, which suggests that they possess a common component which could be X. A sensitivity to azide naturally raises the possibility that a haematin compound is involved, although against this it should be noted that the inhibition was a slow reaction in contrast with the rapid reaction usually occurring between haematin compounds and azide. It is possible that X is mitochrome (Polis & Shmukler, 1954), but all attempts to demonstrate its action in our preparations failed. However, we must await further details of the preparation and properties of mitochrome before we can settle this point.

The ATPase activity at pH 9·4 is also inhibited by azide, although it is not stimulated by DNP. One possibility is that this enzyme involves X, but not I. It would be expected that reaction (3) of the new scheme would occur in two steps, e.g.:

$$X \sim I + H_3PO_4 \rightleftharpoons X \sim P + I \qquad (3a)$$

$$X \sim P + ADP \rightleftharpoons X + ATP \qquad (3b)$$

Some reaction of this type appears to be necessary to explain the rapid exchange between the oxygen atoms of inorganic phosphate and water catalysed by liver mitochondria (Cohn, 1953; Cohn & Drysdale, 1955; Boyer, Falcone & Harrison, 1954; Robertson & Boyer, 1955; Luchsinger, Falcone & Reynard, 1955). The hydrolysis of ATP and the lack of any effect of DNP would be explained if, at pH 9·4, $X \sim P$ reacts with water much more rapidly than with any of the inhibitors.

The mechanism of the stimulation of the latent ATPase activity by ageing and freezing the mitochondria is not clear, but the pH–activity curves suggest that the same enzymes (and therefore the same inhibitors) are involved. It seems likely then that hydrolysis of $X \sim I$ also occurs with these preparations.

SUMMARY

1. The addition to liver mitochondria of inhibitors of the respiratory chain (cyanide, antimycin, malonate), or the removal or inactivation of certain components (diphosphopyridine nucleotide, cytochrome *c*, the 2:3-dimercaptopropanol-labile factor), or the reduction of all components of the respiratory chain by the addition of cyanide and substrate, did not cause any inhibition of the adenosine triphosphatase activity. It appears very unlikely then that a member of the respiratory chain is directly involved in the hydrolysis of adenosine triphosphate (ATP).

2. When azide was added to liver mitochondria, the rate of hydrolysis of ATP was first stimulated (particularly at pH 6) and then inhibited. In the presence of dinitrophenol, the hydrolysis of ATP was inhibited over the whole pH range studied (5·5–10).

3. Calcium stimulated at acid pH values (maximum at pH 6·5) and inhibited at higher pH values.

4. *p*-Chloromercuribenzoate activated the ATPase at all pH values between 5·5 and 9, probably because of a general damage of the mitochondrial structure. In the presence of dinitrophenol the mercurial inhibited between pH 6 and 8 but stimulated at pH 9.

5. Fluoride has little effect on the ATPase activity of aged and frozen mitochondria, but in the presence of dinitrophenol inhibited the activity of normal mitochondria between pH 6 and 8, and stimulated at pH 9.

6. Guanidine, on the other hand, inhibited the systems in aged and frozen mitochondria more than those in normal mitochondria activated by dinitrophenol.

7. Albumin had no effect on the ATPase activity of aged and frozen mitochondria, or on mitochondria activated by azide, but markedly inhibited the activity in the presence of dinitrophenol, especially at alkaline pH values.

8. Dithionite strongly activated the ATPase of aged and frozen mitochondria, with a maximum at pH 7·5.

We wish to thank Mrs M. Jong-Tuynman for her skilled assistance in these experiments.

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A Comparison of the Properties of Mitochondria Isolated from Liver and Heart

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It is now well established that in all animal cells which have been examined the mitochondria are the site of the main intracellular respiratory enzyme systems which are responsible for the synthesis of adenosine triphosphate (ATP). The uniformity of structure, composition and properties of mitochondria isolated from a wide variety of cells is most striking. Nevertheless, some differences might be expected between mitochondria of tissues which differ so much in form and function as the liver and heart muscle. A careful study and correlation of the differences between the mitochondria from the two tissues might possibly throw light on the biochemical mechanisms in the two granules.

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Chance (1956) has recently drawn attention to some differences between the properties of liver mitochondria, as isolated in his laboratory, and heart-muscle mitochondria (sarcosomes) isolated by ourselves. Chance does not consider the possibility that these differences reflect a true difference between the mitochondria in the two tissues. On the contrary, he concludes that the differences are due to the fact that the sarcosomes which we used have been damaged in the course of their preparation.

It is the purpose of the present paper to compare in some detail the properties of the mitochondria isolated from the two tissues. The heart sarcosomes are indeed qualitatively similar to the liver mitochondria, but quantitative differences were observed. An attempt has been made to determine which of these differences can be ascribed to damage

of the sarcosomes and which represent a true difference between the two types of mitochondria *in vivo*.

Part of this work has already appeared in preliminary form (Holton, 1956).

METHODS

Mitochondrial preparations

Liver mitochondria. These were prepared as described in a previous paper (Myers & Slater, 1957).

Heart sarcosomes. Unless otherwise stated, these were prepared from rat heart, according to the procedure described by Cleland & Slater (1953), with 0·21 M-sucrose, 0·01 M-ethylenediaminetetra-acetate (EDTA), pH 7·4, as the isolation medium. The EDTA was the disodium salt brought to pH 7·4 with KOH. We are indebted to Miss A. Searle for most of these preparations.

In an attempt to prepare sarcosomes with a lower ATPase activity in the absence of 2:4-dinitrophenol (DNP), we have tested other isolation media and prepared the heart homogenates with the Potter–Elvehjem homogenizer in place of grinding by hand with sand in a mortar. For these experiments, the heart was minced as finely as possible in cold isotonic medium with a pair of small scissors. It was necessary to operate the homogenizer at a moderately high speed for 1–2 min. in order to obtain a homogeneous suspension of the heart tissue. More gentle grinding of the heart muscle decreased the yield of sarcosomes considerably and did not appear to improve their quality.

In the initial experiments the heart homogenates were centrifuged for 3 min. at 750–800 *g* and the supernatant fluid centrifuged for 7–10 min. at 6000–8000 *g* in order to sediment the sarcosomes. Later experiments showed that better results were obtained when the homogenate was centrifuged for 3 min. at about 600 *g*, the lowest acceleration required to sediment all the erythrocytes and debris. A considerable portion of the sarcosomes in the supernatant fluid could be recovered by a second centrifuging for 10–15 min. at 600 *g*; however, the yield was improved by centrifuging for 10 min. at about 6000 *g*. In contrast with liver homogenates, the preparations obtained from rat heart contain little microsomal material (cf. Cleland & Slater, 1953) and show no evidence of a fluffy layer under the above conditions. In order to minimize the amount of soluble protein associated with the sedimented sarcosomes, we have used large volumes of medium (20–40 ml./rat heart) in the isolation procedure and decanted the supernatant fluid as completely as possible after sedimenting the sarcosomes. The sediment was resuspended in cold isotonic medium with the aid of the homogenizer.

Enzymic tests

Respiratory rates and P:O *ratios.* The rate of O_2 uptake at 25°, expressed as μl. of O_2/mg. of protein/hr. (Q_{O_2}), and the P:O ratios were determined as described by Slater & Holton (1954).

ATPase activity. This was measured at different pH values as described by Myers & Slater (1957).

Analyses

Protein content. This was determined by the biuret method, as described by Cleland & Slater (1953).

Calcium. This was determined as described by Slater & Cleland (1953).

Total iron. This was determined by the phenanthroline method (Mahler & Elowe, 1954) after wet ashing with HNO_3.

Both the calcium and iron determinations were carried out by Mrs A. J. Haarbrink-Haitsma.

Measurement of the difference spectrum of sarcosomes

(*a*) *Optical arrangement.* The sarcosome suspension, containing 6–10 mg. of protein/ml., was contained in a 1 mm. silica cuvette mounted directly in front of the entry slit of the monochromator of the Hilger Uvispek spectrophotometer, model H 700/306. It was illuminated by light from the hydrogen lamp supplied with this instrument. The quartz prism was mounted in the monochromator, and wavelength calibration was carried out with the hydrogen-emission lines at 486·1 mμ and 656·3 mμ. Light emerging from the exit slit was measured by a photomultiplier (E.M.I. Type 6255 B with quartz window). In order to measure at high sensitivity the small changes of extinction due to respiratory catalysts, most of the photomultiplier current was opposed by a current variable at will, and the balance was amplified. The amplified current was recorded by an Evershed and Vignoles Ltd. quick-response pen-writing recording milliammeter. Responses of the recorder were calibrated in terms of changes of extinction by using a mechanical shutter interposed between the light source and the cuvette. This shutter could be moved into the light beam into a series of reproducible positions by means of a micrometer head. During the course of each experiment, while the sarcosome suspension was contained in the cuvette and was in its anaerobic steady state, the response of the recorder was measured for a series of shutter positions. This series was then repeated, and the photomultiplier current measured at each position. From these data the extinction changes corresponding to the recorder responses were readily calculated. The calibration was repeated at several different values of opposing current so that the sensitivity of the recording arrangement at any given value of opposing current was calculable. Full details of this apparatus will be described elsewhere (F. A. Holton, in preparation).

(*b*) *Aeration of suspensions.* The sarcosome suspension was withdrawn from the bottom of the cuvette by means of a fine plastic tube. After passing through a roller pump this tube terminated about 5 mm. above the meniscus of the suspension contained in the cuvette, which was only partly full. During operation of the pump, the suspension circulated at a rate of 1–3 ml./min. and became aerated as it flowed into the cuvette. To assist the establishment and maintenance of an aerobic steady state controlled by the oxidase activity of the suspension, the air above the meniscus was replaced by O_2 during circulation of the suspension, and the circulating tube was kept at 5–10° by a water jacket. Fig. 9 illustrates that this arrangement resulted in an aerobic steady state, which persisted for 15 sec. after circulation had ceased, after which the record returned to the value characteristic of the anaerobic steady state. This illustrates that the aerobic steady state observed was characteristic of the activity of the respiratory enzymes. If the steady response during circulation had been due to a degree of oxygenation controlled by the rate of aeration, the record would have returned to the level of the anaerobic steady state immediately after the flow was stopped.

RESULTS

Respiratory rates and P:O *ratios*

The rates of respiration and the P:O ratios with liver and heart mitochondria oxidizing α-oxoglutarate, succinate and β-hydroxybutyrate are compared in Table 1. Two rates of respiration are given: (*a*) the actual initial rates measured in the determination of the P:O ratios; (*b*) rates which more closely represent the capacity of the mitochondria to oxidize the substrate. The latter rates were measured in the absence of fluoride, which inhibits all the oxidase systems, especially succinic dehydrogenase and the oxidation of β-hydroxybutyrate by heart sarcosomes. A high concentration of succinate (0·06 M) was used in both measurements with the heart sarcosomes, since the rate of respiration is much greater in the presence of high concentrations and the P:O ratio is not affected. With liver mitochondria, the high concentration was also used in method (*b*), in order to measure the true activity of the system oxidizing succinate; however, a lower concentration was used for the measurement of the P:O ratio, since the ratio was thereby increased (K. Minnaert, unpublished observations), and because most other workers studying liver mitochondria have used low succinate concentrations.

The values of Q_{O_2} in columns (*b*), Table 1, represent approximately the activity of the one-step oxidations. With α-oxoglutarate, malonate was added in an amount (0·01 M) which nearly completely suppresses the oxidation of succinate (Slater & Holton, 1954). With succinate, no inhibitor of malate oxidation could be used, but it is probable that under the conditions of high succinate concentration the activity of the succinic oxidase system is so high that the malic oxidase system would be unable to compete successfully for common carriers in the respiratory chain. However, under the conditions in which the P:O ratio of liver mitochondria oxidizing succinate was measured, when the succinic dehydrogenase was operating at much below its full activity (presumably because of the presence of inhibitory oxaloacetate), a considerable proportion of the O_2 uptake probably represents oxidation beyond the malate stage. The Q_{O_2} for liver mitochondria oxidizing β-hydroxybutyrate undoubtedly represents the activity of the single-step oxidation, since acetoacetate is not oxidized by these preparations (Lehninger & Smith, 1949; Walker, 1954). It seems that heart sarcosomes oxidize β-hydroxybutyrate beyond the stage of acetoacetate (Beechey, 1957), but the activity compared with the other substrates is very low.

Table 1 also includes two calculations for each substrate of Q_P, the rate of phosphorylation (μl. of P/mg. of protein/hr.), calculated from each Q_{O_2} by the expression $Q_P = Q_{O_2} \times (P:O) \times 2$. The same value of the P:O ratio was used in the two calculations. Thus, the values of Q_P in calculation (*b*) represent the theoretical rate of phosphorylation, assuming that the P:O ratios were maintained under the conditions in which the maximum Q_{O_2} values were observed. This assumption is probably not valid for liver mitochondria oxidizing succinate.

The other components of the reaction mixture and the procedure used in the determinations of the P:O ratios were the same as previously described (Slater & Holton, 1954), except that a lower concentration of fluoride was used in some measurements. The values reported in Table 1 for liver mitochondria are similar to those obtained by Copenhaver & Lardy (1952), but the P:O ratio with α-oxoglutarate is somewhat lower, and that with succinate appreciably higher.

Table 1. *Rates of respiration and* P:O *values of rat-liver mitochondria and heart sarcosomes*

Mitochondria were isolated from rat liver in 0·25 M-sucrose. Sarcosomes were isolated from rat heart in 0·21 M-sucrose, 0·01 M-EDTA, pH 7·4, by the procedure of Cleland & Slater (1953).

In all cases the reaction mixture contained potassium phosphate, pH 7·4 (0·03 M); glucose (0·02 M); cytochrome *c* ($2–5 \times 10^{-5}$ M); adenosine monophosphate (6×10^{-4} M); adenosine diphosphate (6×10^{-4} M); $MgCl_2$ (0·005 M); EDTA (0·001–0·002 M); hexokinase. Substrate concentrations were: α-oxoglutarate (0·01 M); succinate [0·06 M, except under condition (*a*) for liver mitochondria, when it was 0·006 M]; β-hydroxybutyrate (DL) (0·015 M). Malonate (0·01 M) was used only with α-oxoglutarate. Fluoride concentrations: condition (*a*): with α-oxoglutarate and β-hydroxybutyrate, 0·04 M for heart sarcosomes and 0·02 M for liver mitochondria, with succinate, 0·02 M in both cases; condition (*b*): no fluoride with any substrate.

Values given are the means. $Q_{O_2} = \mu$l. of O_2/mg. of protein/hr. Figures in parentheses after P:O are the no. of expts.

$$Q_P = 2 \times Q_{O_2} \times [P:O]$$

	Heart sarcosomes		Liver mitochondria	
	(*a*)	(*b*)	(*a*)	(*b*)
α-Oxoglutarate				
Q_{O_2}	91	148	22	30
P:O	2·86 (19)	—	3·06 (9)	—
Q_P	520	850	135	183
Succinate				
Q_{O_2}	188	327	25·7	162
P:O	0·98 (17)	—	2·21 (8)	—
Q_P	367	650	113	(716)*
β-Hydroxybutyrate				
Q_{O_2}	11·6	25	32	32
P:O	1·97 (4)	—	2·58 (6)	—
Q_P	46	98	165	165

* See text for discussion of this value.

The most important differences between the heart sarcosomes and liver mitochondria shown in Table 1 are:

(1) The much greater respiratory activity of heart sarcosomes, which oxidize α-oxoglutarate five times and succinate twice as fast as liver mitochondria. The activities towards β-hydroxybutyrate do not differ appreciably.

(2) The much lower P:O ratios obtained with heart sarcosomes oxidizing succinate and β-hydroxybutyrate.

Because of the higher rate of respiration, the heart sarcosomes are able to synthesize ATP at a much greater rate (Q_P) than liver mitochondria.

Hydrolysis of adenosine triphosphate

Fig. 1 shows the pH–activity curves for the hydrolysis of ATP by liver mitochondria and heart sarcosomes (isolated in 0·21 M-sucrose, 0·01 M-EDTA, pH 7·4, by the procedure of Cleland & Slater, 1953), both in the absence and presence of DNP (10^{-4} M) and in the absence and presence of Mg^{2+} ions (0·003 M).

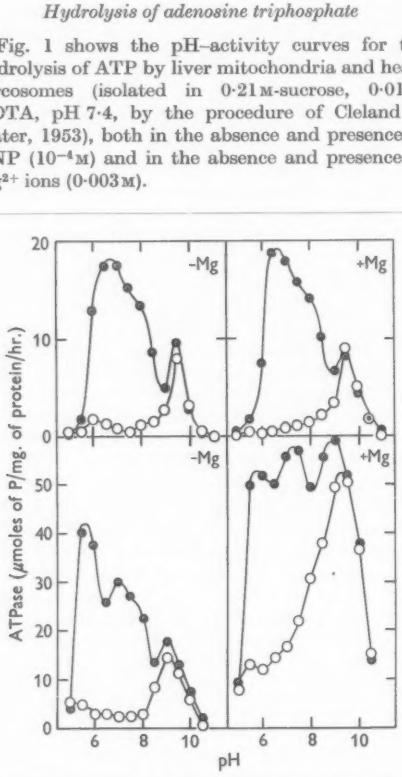

Fig. 1. Effect of DNP and Mg^{2+} ions on the hydrolysis of ATP by (upper curves) rat-liver mitochondria (isolated in 0·25 M-sucrose) and (lower curves) heart sarcosomes (isolated in 0·21 M-sucrose, 0·01 M-EDTA, pH 7·4). The reaction mixture contained 0·075 M-KCl, 0·05 M-sucrose, 0·05 M-tris buffer, 0·001 M-EDTA, 0·002 M-ATP, 0·1 mg. (liver) or 0·05 mg. (heart) of mitochondrial protein/ml., with or without 0·003 M-$MgCl_2$ as shown. ○, Absence of DNP; ●, addition of 10^{-4} M-DNP.

In the absence of Mg^{2+} ions, the shapes of the curves with the two types of preparation are similar both in the absence and in the presence of DNP. In both cases there is little activity under acid or neutral conditions in the absence of DNP, but considerable activity was found in alkaline solution, with optima at pH 9·4 (liver) and 9·2 (heart). DNP markedly activates the ATPase in both preparations below pH 9, but has no appreciable effect above pH 9. The degree of stimulation of the ATPase brought about by DNP is similar in the two preparations.

The two preparations differ in their response to Mg^{2+} ions, which have little effect on liver mitochondria, but markedly activate the ATPase of the heart sarcosomes, especially in the absence of DNP. Consequently, in the presence of Mg^{2+} ions, the degree of stimulation of the ATPase brought about by the addition of DNP at neutral pH is much less with heart sarcosomes than with liver mitochondria. Average rates of hydrolysis of ATP at pH 7·4 for the two types of mitochondria are compared in Table 2.

In a previous paper (Myers & Slater, 1957), it was shown that four peaks (at pH 6·3, 7·4, 8·5 and 9·4) could be obtained in the pH–activity curves of the ATPase of liver mitochondria, under various conditions. The first three peaks could be demonstrated by the addition of different concentrations of DNP to freshly prepared mitochondria, and the fourth was already present in the absence of DNP. In the presence of DNP (10^{-4} M), the pH–activity curve with heart sarcosomes is similar to that given by liver mitochondria, except that the alkaline system is much more dominant in the sarcosomes, in the

Table 2. *Comparison of the ATPase activities of rat-liver mitochondria and heart sarcosomes at* pH 7·4.

Mitochondria and sarcosomes were isolated as in Table 1. The reaction mixture contained 0·075 M-KCl, 0·108 M-sucrose, 0·001 M-EDTA and 0·002 M-ATP with or without further additions of $MgCl_2$ and DNP as noted below. The mixture was incubated for 15 min. at 20° and pH 7·4 after addition of 0·08–0·3 mg. of mitochondrial protein/ml. or 0·05–0·1 mg. of sarcosomal protein/ml. The average rates of hydrolysis of ATP observed with eleven preparations of liver mitochondria and three preparations of heart sarcosomes are given.

DNP (M)	$MgCl_2$ (M)	Average rates of hydrolysis of ATP (μmoles of P/mg. of protein/hr.) Liver mitochondria	Heart sarcosomes
0	0	1·1	1·2
10^{-4}	0	13·1	31·2
0	0·003	0·75	27·7
10^{-4}	0·003	11·9	54·0

presence of Mg^{2+} ions. When DNP was added, three clear peaks were obtained with the heart sarcosomes at pH 5·7, 7·1–7·3 and 9–9·2 respectively, both in the absence and presence of Mg^{2+} ions. A peak at pH 8·2 is shown by plotting the difference curve (+Mg^{2+} ions *minus* −Mg^{2+} ions) in the absence of DNP (Fig. 2).

The specific activity of the heart sarcosomes at neutral pH in the presence of Mg^{2+} ions and DNP is about five times that of liver mitochondria. In the absence of DNP, liver mitochondria can be activated by ageing, and especially by freezing and thawing after ageing. The activity after this treatment is, in fact, much greater than obtained with unaged mitochondria in the presence of DNP (Myers & Slater, 1957). Fig. 3 shows that heart sarcosomes (isolated in 0·25 M-sucrose, without EDTA) behave in the same way, and that the pH–activity curves of the two preparations after ageing, freezing and thawing are very similar, both being dominated by the peaks at pH 9, but also showing inflexions at about pH 7. The specific activity of the heart sarcosomes treated in this way was double that of the liver mitochondria.

It is clear that there are striking resemblances between the systems hydrolysing ATP in the two types of mitochondria. The chief difference is the response of the system in the sarcosomes to Mg^{2+} ions, particularly at alkaline pH values.

In the absence of DNP but presence of Mg^{2+} ions, the hydrolysis of ATP by heart sarcosomes was not inhibited by 0·06 M-NaF. In this respect this ATPase behaves like that in aged mitochondria, frozen mitochondria or in the Keilin & Hartree heart-muscle preparation (Myers & Slater, 1957). However, the increment of the ATPase activity of heart sarcosomes brought about by the addition of 10^{-4} M-DNP was almost completely inhibited by 0·06 M-NaF. The DNP-stimulated ATPase activity of liver mitochondria is likewise inhibited by 0·06 M-NaF.

Attempts were made to isolate heart sarcosomes more closely resembling liver mitochondria by varying the procedure of isolation. Similar results were obtained by grinding the heart in a mortar with sand, as in the earlier procedure (Cleland & Slater, 1953), or by using a Potter–Elvehjem homogenizer (Fig. 4). In experiments in which various isolation media were tried, the Potter–Elvehjem homogenizer was used. A series of glass homogenizers with different clearances between the tube and pestle was tested, but none of these provided better preparations than those obtained with the standard

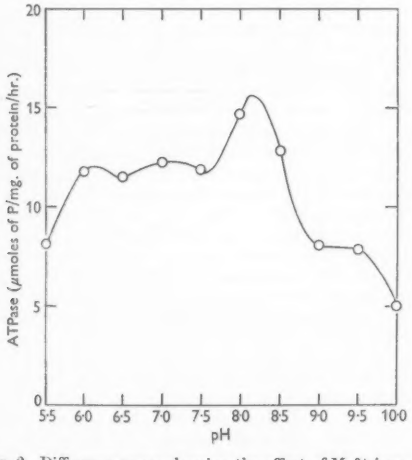

Fig. 2. Difference curve showing the effect of Mg^{2+} ions on the hydrolysis of ATP by rat-heart sarcosomes (isolated in 0·23 M-sucrose, 0·005 M-ATP, pH 7·4). Reaction mixture: KCl (0·075 M); EDTA (6×10^{-4} M); ATP (0·002 M); tris buffer (0·05 M); sucrose (0·05 M); sarcosomes (0·18 mg. of protein/ml.). The ATPase activity plotted is activity measured in the presence of 0·003 M-$MgCl_2$ *minus* activity measured in the absence of added Mg^{2+} ions.

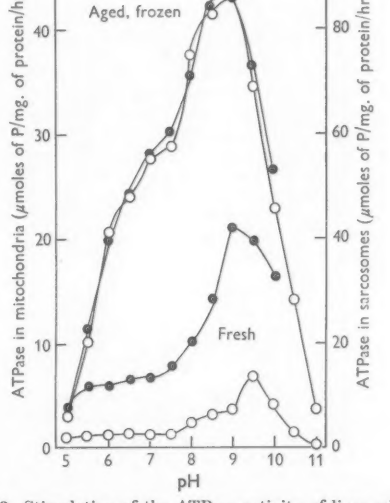

Fig. 3. Stimulation of the ATPase activity of liver mitochondria (○) and of heart sarcosomes (●) by ageing (2 hr. at 30°) followed by freezing and thawing (upper curves). The activities of the freshly prepared mitochondria (○) and sarcosomes (●) are shown in the lower curves. Both preparations were isolated in 0·25 M-sucrose. Reaction mixture: 0·075 M-KCl, 0·05 M-sucrose, 0·05 M-tris buffer, 0·002 M-ATP and 0·001 M-$MgCl_2$.

homogenizer with a polytetrafluoroethylene (Teflon) pestle. The degree of stimulation of the ATPase activity by DNP was not increased appreciably when the isolation was carried out under anaerobic conditions.

In a series of experiments with different isolation media, it was found that the following all gave similar results: (1) 0·25 M-sucrose (cf. Plaut & Plaut, 1952; Maley & Plaut, 1953); (2) 0·21 or 0·225 M-sucrose, 0·01 M-EDTA, pH 7·4 (Cleland & Slater, 1953); (3) 0·24 M-sucrose, 0·5% plasma albumin (Sacktor, 1954; Lewis & Slater, 1954); (4) 0·225 M-sucrose, 0·01 M-α-oxoglutarate, pH 7·4; (5) 0·225 M-sucrose, 0·01 M-citrate, pH 7·4, 6·8 or 6·2; (6) 0·23 M-sucrose, 0·005 M-adenosine diphosphate (ADP), pH 7·4; (7) 0·23 M-sucrose, 0·005 M-adenosine monophosphate, pH 7·4; (8) 0·15 M-KCl; (9) 0·135 M-KCl, 0·02 M-phosphate, pH 7·4 (Cleland & Slater, 1953); (10) 0·121 M-KCl, 0·02 M-phosphate, 5×10^{-4} M-ATP, pH 7·4 (Montgomery & Webb, 1956). In all cases, the ATPase activity of the sarcosomes at pH 7·4 in the presence of Mg^{2+} ions was increased approximately twofold by the addition of 10^{-4} M-DNP. The degree of stimulation by DNP was significantly smaller when the concentration of sucrose in the isolation medium was increased to 0·88 M or when the sarcosomes were isolated in 0·44 M-sucrose, 0·01 M-citrate, pH 6·2 as recommended by Witter, Watson & Cottone (1955). On the other hand, the ATPase activity at pH 7·4 in

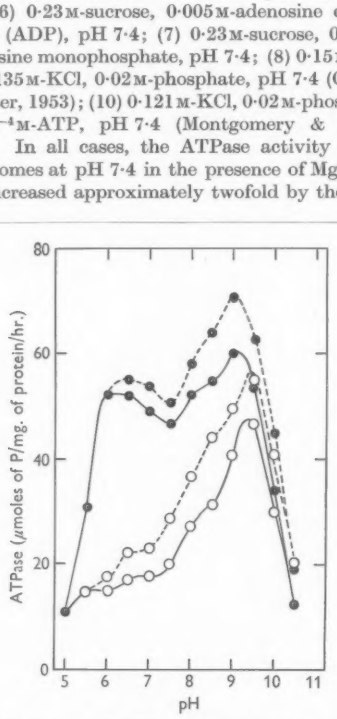

Fig. 4. Hydrolysis of ATP by two preparations of rat-heart sarcosomes. The reaction mixture is the same as that given for Table 3. Sarcosome preparations were isolated from rat heart in 0·21 M-sucrose, 0·01 M-EDTA, pH 7·4. One preparation was isolated by the standard procedure of Cleland & Slater (1953) and its ATPase activities were measured in the absence (○ - - - ○) and presence (●—●) of 10^{-4} M-DNP. The other preparation was isolated simultaneously and treated in the same way but all homogenizing and mixing operations were carried out with a Potter–Elvehjem homogenizer as described in the text; ATPase activities were measured in the absence (○—○) and presence (● - - - ●) of 10^{-4} M-DNP.

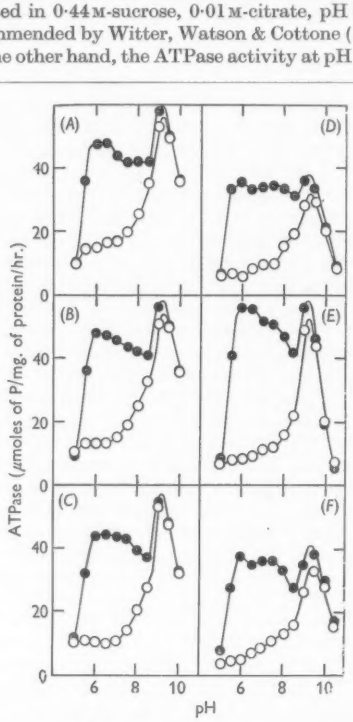

Fig. 5. Hydrolysis of ATP by rat-heart sarcosomes isolated in various media. The reaction medium was the same as in Table 3 and the ATPase activities of the sarcosome preparations were measured in the absence (○) and presence (●) of 10^{-4} M-DNP. Graphs (*A*), (*B*) and (*C*) represent three preparations which were isolated simultaneously in different media from the same rat heart; the heart homogenates were centrifuged for 3 min. at 600 *g* and the sarcosomes were recovered from the supernatant fluids by centrifuging for 7·5 min. at 6000 *g*. Graphs (*D*), (*E*) and (*F*) represent different preparations in which the sarcosomes were recovered from the supernatant fluid by a second centrifuging at 600 *g* for 10–15 min. In all cases the sarcosomes were resuspended in the same medium as used for the isolation procedure. The following media were used: (*A*) 0·25 M-sucrose; (*B*) 0·15 M-KCl; (*C*) 0·135 M-KCl, 0·005 M-ATP, pH 7·4; (*D*) 0·23 M-sucrose, 0·005 M-ATP, pH 7·4 (average of three experiments); (*E*) 0·23 M-sucrose, 0·005 M-ATP, pH 7·4 [this preparation differs from those given in graph (*D*) in that small rats weighing 50 g. were used in place of adult rats weighing approx. 250 g.]; (*F*) 0·1 M-KCl, 0·05 M-tris buffer, 0·001 M-ATP, 0·005 M-$MgCl_2$, 0·001 M-EDTA, pH 7·4 (cf. Chappell & Perry, 1954) (average of two experiments).

the presence of Mg^{2+} ions was increased by DNP about 3·3 times when the sarcosomes were isolated in an isotonic medium containing ATP. This result was obtained with the following isolation media: (1) 0·23 M-sucrose, 0·005 M-ATP, pH 7·4, 6·8 or 6·2; (2) 0·23 M-sucrose, 0·005 M-ATP, 0·005 M-$MgCl_2$, pH 7·4; (3) 0·21 M-sucrose, 0·005 M-ATP, 0·01 M-α-oxoglutarate, pH 7·4; (4) 0·135 M-KCl, 0·005 M-ATP, pH 7·4; (5) 0·1 M-KCl, 0·05 M-2-amino-2-hydroxymethylpropane-1:3-diol (tris) buffer, 0·001 M-ATP, 0·005 M-$MgCl_2$, 0·001 M-EDTA, pH 7·4 (Chappell & Perry, 1954). The sarcosomes isolated in all of these media appeared to have similar ATPase activities. Typical results obtained with sarcosomes isolated in media with and without ATP are shown in Fig. 5.

Further experiments showed that the sarcosomes present in the homogenates of rat heart were not completely homogeneous and that those with the lowest ATPase activity in the presence of Mg^{2+} ions could be centrifuged down at low speeds. A typical experiment on the differential centrifuging of homogenates in 0·25 M-sucrose is shown in Table 3; similar results were obtained with homogenates in sucrose + EDTA, sucrose + ATP and the medium of Chappell & Perry (1954). A considerable fraction of the sarcosomes in heart homogenates was sedimented by centrifuging at 600 *g*; on the other hand, the major portion of the mitochondria in liver homogenates was sedimented only at about 4000 *g*.

The ATPase activity of the clear supernatant fluid obtained after centrifuging the heart homogenate at 20 000 *g* was still stimulated by DNP. In

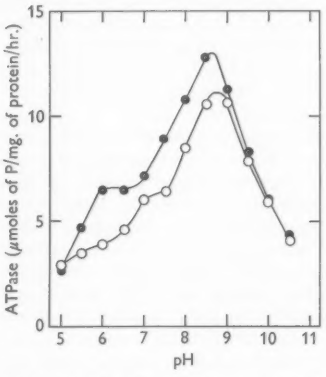

Fig. 6. Hydrolysis of ATP by the supernatant fluid from a rat-heart homogenate after centrifuging at 20 000 *g*. The reaction medium was the same as in Table 3; the ATPase activities of the supernatant fluid were measured in the absence (○) and presence (●) of 10^{-4} M-DNP.

Table 3. *Fractionation of liver and heart homogenates by differential centrifuging*

Homogenates of rat liver and rat heart in 0·25 M-sucrose were prepared with the Potter–Elvehjem homogenizer. The homogenates were centrifuged for 5 min. at 600 *g*, and approx. 75% of the supernatant fluids was decanted into other centrifuge tubes; the remainder of the supernatant was discarded together with the sediment. The retained supernatant from the heart homogenate contained a total of 26·6 mg. of protein, that from the liver homogenate contained a total of 249 mg. of protein in about 12 ml. of sucrose solution. These supernatant fluids were centrifuged again for 10 min. at 600 *g*, the sediments were kept for determination of the ATPase activity, and the supernatants were centrifuged for 10 min. at 1200 *g*. This procedure was repeated at successively higher speeds up to 20 000 *g*. The supernatant fluids were decanted completely each time but no attempt was made to separate the microsomal components from the mitochondria which were sedimented at high speeds. The reaction medium used for the ATPase assay contained KCl, 0·075 M; sucrose, 0·05 M; tris–acetate buffer, pH 7·5, 0·05 M; $MgCl_2$, 0·001 M; EDTA, 6×10^{-4} M; DNP (where shown), 10^{-4} M. Reaction occurred for 15 min. at 20°.

Fraction of the initial supernatant		Protein content (% of total protein)	ATPase activity (μmoles of P/mg. of protein/hr.)		Stimulation of the ATPase activity by DNP (%)
			$MgCl_2$	$MgCl_2$ + DNP	
Rat heart					
Sediment	600 *g*	9	16·0	41·5	159
Sediment	1 200 *g*	7	18·1	42·2	133
Sediment	4 000 *g*	5	28·5	48·3	69
Sediment	8 000 *g*	2	34·9	48·0	38
Sediment	20 000 *g*	3	36·5	51·8	41
Supernatant	20 000 *g*	74	6·4	8·8	37
Rat liver					
Sediment	600 *g*	13	2·6	11·5	340
Sediment	1 200 *g*	6	1·9	14·4	660
Sediment	4 000 *g*	10	1·5	12·3	720
Sediment	8 000 *g*	5	1·8	9·8	440
Sediment	20 000 *g*	4	1·9	5·0	163
Supernatant	20 000 *g*	62	1·1	1·1	0

confirmation of the data given by Lardy & Wellman (1953), the corresponding turbid supernatant from liver homogenates did not show this effect. The pH–activity curve obtained with the supernatant from a heart homogenate was similar to that obtained with intact sarcosomes, although the specific ATPase activity is relatively low because of the presence of soluble proteins (Fig. 6). This would suggest that the supernatant fluid may contain small fragments from the sarcosomes which have been broken during the homogenization of the heart muscle.

The differences in the results obtained with liver mitochondria and heart sarcosomes are probably not due to an inherent instability of the sarcosomes in the isotonic sucrose. The maximum rate of hydrolysis of ATP at pH 7·4 falls slowly when the isolated sarcosomes are allowed to stand in the same medium for 3–4 hr. at 0°, but the degree of stimulation caused by DNP was only slightly decreased (Table 4). The activity of the α-oxoglutaric oxidase of heart sarcosomes in sucrose solution is also stable for 1–3 hr. at 0°, although it deteriorates rapidly at 25° unless EDTA is added to the medium (Slater & Cleland, 1953). Other experiments have shown that the ATPase activity of the sarcosomes is relatively stable when the whole homogenate is allowed to stand for 3 hr. at 0°. It is concluded therefore that any damage which might have been caused to the sarcosomes must have occurred during the preparation of the heart homogenate and not at a later stage in the isolation procedure.

Our attempts to circumvent damage of this type have failed to produce heart sarcosomes with an ATPase activity similar to that of liver mitochondria. However, when young rats weighing 30–40 g. were used in place of the usual adult rats weighing about 250 g., a somewhat greater degree of stimulation by DNP was sometimes found. Fig. 5 shows the ATPase activities of the sarcosomes isolated at 600 *g* in the sucrose–ATP medium from the heart muscle of these young rats. The increase in the degree of stimulation by DNP under these conditions may be due to the ease with which this heart muscle can be homogenized.

Sarcosomes isolated from mouse heart behaved very similarly to those from rat heart. However, guinea-pig-heart sarcosomes had a considerably lower ATPase activity (16 μmoles/mg. of protein/hr. at pH 7·4, in the presence of Mg^{2+} ions and DNP,

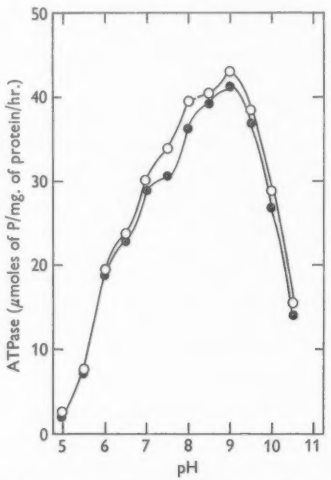

Fig. 7. Hydrolysis of ATP by the Keilin & Hartree (1947) heart-muscle preparation. The reaction medium was the same as in Fig. 2. Average values are given for three preparations isolated from horse heart by the standard procedure of Keilin & Hartree (●) and for three preparations isolated by high-speed centrifuging (○) (cf. Slater, 1949).

Table 4. *ATPase activities of heart sarcosomes after standing at 0°*

Sarcosomes were isolated from rat-heart homogenates in 0·25 M-sucrose or in 0·23 M-sucrose, 0·005 M-ATP (pH 7·4) by centrifuging at 600 *g* for 10 min. as described under Methods. They were resuspended in the same medium as that used in the isolation procedure and allowed to stand at 0°. The ATPase activities at pH 7·5 were determined as in Table 3.

Medium	Time at 0° (hr.)	ATPase activity (μmoles of P/mg. of protein/hr.) $MgCl_2$	ATPase activity (μmoles of P/mg. of protein/hr.) $MgCl_2$ + DNP	Stimulation of the ATPase activity by DNP (%)
Sucrose	0·2	14·9	34·0	128
	3·5	13·8	29·7	115
Sucrose	0·2	15·6	43·9	181
	4·0	15·2	40·6	167
Sucrose + ATP	0·2	10·4	33·7	224
	4·0	9·1	27·3	200
Sucrose + ATP	0·2	9·6	29·5	208
	4·0	9·1	27·2	199

compared with 54 μmoles of P/mg. of protein/hr. for rat heart) and the degree of stimulation by DNP at pH 7·4 was often also higher. There was no difference in the ATPase activities of mitochondria from rat and guinea-pig liver.

The Keilin & Hartree (1947) horse-heart-muscle preparation, which consists of sarcosomal fragments, had half the ATPase activity of aged and frozen rat-heart sarcosomes; the pH–activity curves were very similar (cf. Fig. 7 and Fig. 3). Similar results were obtained whether the sarcosomal fragments were sedimented by high-speed centrifuging at neutral pH or by low-speed centrifuging at pH 5·7, as in the procedure of Keilin & Hartree (1947).

The ATPase activity of the Keilin & Hartree preparation was much more stable than that of aged and frozen liver mitochondria (Myers & Slater, 1957), there being little loss after storage for 4 days at 2° in 0·1 M-phosphate, pH 7·4, or in 0·235 M-sucrose, 0·01 M-tris, or for 7 days at −18° in the latter medium. The high ATPase activity of the horse-heart preparation was not observed by Keilin & Hartree (1949), who tested the reaction in the absence of added bivalent cations. We have confirmed the low activity under these conditions and shown that Mg^{2+} ions and a number of other cations activate the reaction (Myers & Slater, 1957). The sarcosomal fragments which constitute the Keilin & Hartree heart-muscle preparation are probably similar to the mitochondrial fragments prepared from liver by Kielley & Kielley (1953).

Respiratory control

We have previously reported an experiment (Slater & Holton, 1953) designed in connexion with an investigation of the adenine nucleotide specificity of oxidative phosphorylation, in which we showed the following relative rates of oxidation with α-oxoglutarate as substrate: no adenine nucleotide, no hexokinase, 1; no adenine nucleotide, with hexokinase, 1·9; ATP, no hexokinase, 2·5; ADP, with hexokinase, 5·2. The stimulation by hexokinase shows that, as with liver mitochondria (Lardy & Wellman, 1952), the rate of oxidation of α-oxoglutarate by heart sarcosomes is limited by the concentration of phosphate acceptor.

The degree of respiratory control obtained with liver mitochondria and heart sarcosomes has now been studied in detail. The respiratory-control index is defined as [rate of respiration in the presence of DNP or phosphate acceptor (ADP or glucose)]/(rate of respiration in the presence of 10^{-4} M-ATP). We used a low concentration of ATP in the control measurement, since in its absence the oxidase systems were often irreversibly inactivated after a short time. Higher concentrations were not used, since they supplied sufficient ADP to maintain an appreciable concentration of phosphate acceptor (cf. Chance & Williams, 1955*a*). Under these conditions the rates of O_2 uptake were essentially constant for about 30 min. at 25°, except when DNP was present, when the rate of O_2 uptake often sharply decreased. Two types of heart sarcosomes were used, one isolated in 0·21 M-sucrose, 0·01 M-EDTA, pH 7·4, exactly as in previous papers, and the other isolated in 0·25 M-sucrose, 0·005 M-ATP, pH 7·4.

Before a comparison between the two types of mitochondria could be made, it was necessary to study factors which affected the degree of respiratory control in both types of mitochondria. The following factors were found important:

(1) *Presence of magnesium.* The addition of Mg^{2+} ions increased the respiratory-control index with liver mitochondria and heart sarcosomes, but its effect was much more marked with the former. Qualitatively, the effects of omitting Mg^{2+} ions were also different. With liver mitochondria, the rate of respiration in the absence of acceptor was increased to about the same value as obtained in the presence of acceptor. The latter rate was scarcely affected by omission of Mg^{2+} ions. With heart sarcosomes, however, omission of Mg^{2+} ions did not affect the rate in the absence of phosphate acceptor, but decreased the rate in its presence. The different effects of Mg^{2+} ions in the absence of acceptor suggest that it is necessary for stabilization of liver mitochondria (Baltscheffsky, 1956), but not for heart sarcosomes.

(2) *Presence of fluoride.* In both liver mitochondria and heart sarcosomes, the respiratory-control index was substantially higher in the presence of fluoride (0·012 M).

(3) *Concentration of mitochondria.* Increasing concentration of mitochondria increased the respiratory-control index with both types of preparations (Table 5). With liver mitochondria, this was due both to a decrease in the Q_{O_2} in the absence of phosphate acceptor (or DNP) and to an increase of the Q_{O_2} with increasing concentration of mitochondria, when hexokinase–glucose or ADP was added. This was also observed by Stern & Timonen (1956), who further found that the inactivating effect of dilution could be prevented by the addition of the nuclear fraction, or of a heated nuclear fraction. When DNP was used instead of hexokinase and glucose, the effect of concentration of mitochondria was rather variable, and Table 5 shows an experiment (liver mitochondria II) in which the Q_{O_2} decreased with increasing concentration.

Liver mitochondria had approximately the same respiratory-control index with hexokinase–glucose, DNP, or ADP, but heart sarcosomes gave the highest results with ADP. The concentration of the heart sarcosomes had little effect on the Q_{O_2} in the

absence of acceptor, but the Q_{O_2} in the presence of ADP increased with increasing concentration.

The increase of the Q_{O_2}, in the absence of acceptor, brought about by lowering the concentration of liver mitochondria may be correlated with the greater ATPase activity of these mitochondria in dilute solution (Myers & Slater, 1957).

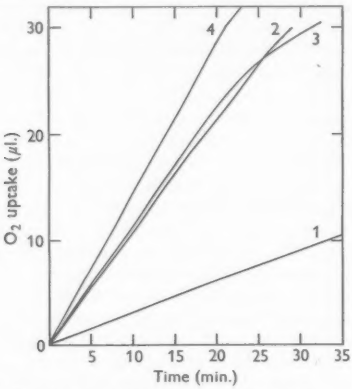

Fig. 8. Respiratory-control index with rat-heart sarcosomes, isolated in 0·23 M-sucrose, 0·005 M-ATP, pH 7·4, and suspended in 0·25 M-sucrose, 0·001 M-ATP, pH 7·4. Reaction mixture: phosphate, pH 7·4 (0·016 M); NaF (0·012 M); $MgCl_2$ (0·006 M); KCl (0·083 M); ATP (10^{-4} M); EDTA (0·001 M); glutamate (DL) (0·007 M); sarcosomes (0·8 mg. of protein/ml.). Reaction vol., 1 ml.; temp., 25°. Curve 1, no further addition; curve 2, 0·02 M-glucose + hexokinase (P:O ratio measured on this flask = 2·13); curve 3, 10^{-4} M-DNP; curve 4, 0·0033 M-ADP.

(4) *Substrate.* In agreement with the results of Lardy & Wellman (1952), a greater respiratory-control index was obtained with glutamate than with α-oxoglutarate, succinate, or β-hydroxybutyrate as substrate.

(5) pH. A higher respiratory-control index was obtained at pH 7·2 than at 6·2 or 6·7.

An experiment demonstrating respiratory control with rat-heart sarcosomes is shown in Fig. 8. In other experiments with guinea-pig-heart sarcosomes, which have a lower ATPase activity, the rate of O_2 uptake showed a sudden decline when all the ADP was phosphorylated to ATP. This also occurred with liver mitochondria (cf. Chance & Williams, 1955*b*).

Diphosphopyridine nucleotide content of mitochondria

The DPN content of liver mitochondria was determined by precipitation of the protein with $HClO_4$, centrifuging and measurement of the DPN content of the neutralized supernatant with alcohol dehydrogenase, as described by Racker (1950). Since Chance & Williams (1955*b*) have found that isolated liver mitochondria contain most of the DPN in the reduced form, and DPNH is unstable in acid solution, the liver mitochondria were incubated for a short time with ADP before addition of the $HClO_4$ in order to oxidize the DPNH (Chance & Williams, 1955*b*). Table 6 shows the effect of time of

Table 5. *Effect of concentration of mitochondria on the respiratory-control index*

The reaction mixture contained phosphate, pH 7·4 (0·016 M); NaF (0·012 M); $MgCl_2$ (0·006 M); KCl (0·083 M); ATP (10^{-4} M); EDTA (0·001 M); glutamate (DL) (0·007 M); DNP (where added, 10^{-4} M); ADP (where added, 0·0027 M); glucose (added with hexokinase, 0·02 M). Temperature, 25°.

		Q_{O_2} (µl. of O_2/mg. of protein/hr.)				
Preparation	Protein (mg./ml.)	ATP (*a*)	ATP + hexokinase (*b*)	ATP + DNP (*c*)	ATP + ADP (*d*)	Respiratory-control index (*b*/*a*), (*c*/*a*) or (*d*/*a*)
Liver mitochondria I	0·67	14·9	32·8	—	—	2·2
	1·34	13·4	50·8	—	—	3·8
	2·68	10·4	52·2	—	—	5·0
Liver mitochondria II	1·0	13·0	—	52·0	—	4·0
	2·0	8·0	—	41·0	—	5·1
	4·0	7·0	—	41·0	—	5·9
Liver mitochondria III	1·15	12·7	—	34·4	—	2·7
	2·30	8·3	—	38·1	—	4·6
Liver mitochondria IV	1·32	12·7	—	—	37·7	3·0
	3·96	10·9	—	—	75·9	7·0
Heart sarcosomes I*	0·30	41	—	—	110	2·7
	0·60	43	—	—	135	3·2
	0·90	37	—	—	132	3·6
Heart sarcosomes II†	0·26	21	—	—	36	1·8
	0·52	23	—	—	52	2·25
	0·78	24	—	—	58	2·4

* Isolated in 0·21 M-sucrose, 0·01 M-EDTA, pH 7·4, by the method of Cleland & Slater (1953).
† Isolated in 0·23 M-sucrose, 0·005 M-ATP, pH 7·4.

incubation on the DPN found. The results show that between 43 and 65 % of the total DPN was not recovered by deproteinizing before the addition of the ADP. The actual percentage of the pyridine nucleotide present in the reduced form in the isolated mitochondria might be greater than these values, since it is not certain that all of the DPNH would be destroyed by the isolation procedure. Pre-incubation with ADP had little or no effect on the amount of DPN extracted from the heart sarcosomes. The value found for sarcosomes isolated in sucrose–EDTA is the same as that obtained by Holton (1955). Sarcosomes isolated in the medium of Chappell & Perry (1954) contained about the same amount of DPN.

Difference spectrum of sarcosomes from 300 to 350 mμ

Although Holton (1955) showed the presence of large amounts of DPN in heart sarcosomes, the difference spectrum (anaerobic *minus* aerobic state) did not include the expected maximum in the region 320–350 mμ when the reducing substrate was β-hydroxybutyrate. This question has now been investigated by the procedure described under Methods.

Heart sarcosomes were suspended in a 0·32-osmolar mixture containing DL-β-hydroxybutyrate, ATP, EDTA and sucrose. Upon aeration of the sarcosome suspension, negative changes of extinction occurred at wavelengths between 300 and 350 mμ with both rat- and rabbit-heart sarcosomes. These changes were reversed by stopping the aeration (Fig. 9). The kinetics of the extinction changes were closely similar to the simultaneous changes recorded at 440–450 mμ, which are mainly due to reactions of cytochrome a_3. Thus they may safely be taken to represent oxidation and reduction of respiratory catalysts.

The difference spectra (anaerobic *minus* aerobic steady states) for two different sarcosome suspensions are shown in Fig. 10. They show a marked peak at about 320 mμ. This is about the same position as that found in liver mitochondria by Chance & Williams (1955*c*, Fig. 1).

Calcium and iron content of mitochondria

Table 7 compares the calcium and iron contents of the two types of mitochondria. For comparison, the calcium content of sarcosomes isolated in 0·25 M-sucrose is given. Sarcosomes isolated by the

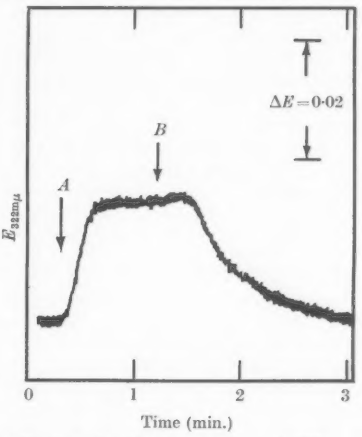

Fig. 9. Kinetics of extinction changes at 322 mμ in an aerated sarcosome suspension. Actual tracing from pen recorder. An upward deflexion represents a decrease of the extinction. Rat-heart sarcosomes, 6·6 mg. of protein/ml.; β-hydroxybutyrate (DL), 0·0085 M; ATP, 0·0079 M; sucrose, 0·27 M; EDTA, 0·001 M. Band width, 4·4 mμ, expts. 56/38. Aeration commenced at arrow *A* and stopped at arrow *B*.

Table 6. *Diphosphopyridine nucleotide content of rat-liver mitochondria and heart sarcosomes*

A volume (0·2 ml.) of a solution containing 0·024 M-ADP, 0·012 M-EDTA, pH 7·4, was added to 2 ml. of mitochondrial or sarcosomal suspension, and the mixture allowed to stand for different times at 22°, with frequent shaking; $HClO_4$ (0·2 ml. of 70 %, v/v) was then added, and the DPN content of the deproteinized extract determined according to the procedure of Holton (1955). The unincubated samples were treated with $HClO_4$ before the addition of ADP and EDTA. A control solution was prepared in the same way, with the substitution of 2 ml. of isolation medium for the mitochondria. This solution was used in the reference cell in the determination of the DPN.

	DPN (μmoles/g. of protein)			
Time of incubation with ADP (min.) ...	0	5	10	15
Preparation				
Liver mitochondria I	2·64	—	4·66	—
Liver mitochondria II	1·84	4·56	4·60	4·44
Liver mitochondria III	1·50	—	4·32	—
Heart sarcosomes I*	6·7	—	—	—
Heart sarcosomes II†	6·1	—	6·4	—

* Isolated in 0·1 M-KCl, 0·05 M-tris, 0·001 M-ATP, 0·005 M-$MgCl_2$, 0·001 M-EDTA, pH 7·4 (Chappell & Perry, 1954).
† Isolated in 0·21 M-sucrose, 0·01 M-EDTA, pH 7·4 (Cleland & Slater, 1953).

method of Cleland & Slater (1953) contain much less calcium, which is bound by the EDTA in the medium (Slater & Cleland, 1953). EDTA does not remove iron from mitochondrial preparations.

Both types of mitochondria contain large amounts of iron, most of which is probably not in the form of haem (cf. Green, 1956). The amount of iron in liver mitochondria corresponds to about 1 mole/50 000 g. of protein.

DISCUSSION

Chance (1956) has recently compared the properties of liver mitochondria, isolated by himself, with those of heart-muscle mitochondria (sarcosomes) isolated by ourselves in the following words: 'In summary, four of Slater's experimental data on his own preparations distinguish them from the liver preparations; their "less marked" respiratory control, their low pyridine nucleotide content, their low P:O value, and the kinetics of cytochrome *b*. All these differences are consistent with the idea that the muscle sarcosomes studied so far by Slater have been somehow damaged in the course of their preparation. Direct evidence of damage to their respiratory-control mechanism is afforded by evidence cited in Lardy's report on the great range of respiratory control obtainable in intact muscle.'

In considering these differences it should be remembered that Chance has not reported any experiments on heart sarcosomes. The sarcosomes referred to by Chance & Williams (1955*c*) were isolated from blowfly thoracic muscle. We have found that these preparations phosphorylate much more poorly than heart sarcosomes (Lewis & Slater, 1954). The only other study of oxidative phosphorylation in heart that we are aware of was made by Maley & Plaut (1953, 1954), who obtained slightly higher P:O ratios than ours with α-oxoglutarate, and considerably higher ratios with succinate. Unfortunately, none of the other properties discussed in this paper have been studied with the sarcosomes isolated by Maley & Plaut, and we have not succeeded in isolating sarcosomes with such high P:O values. In what follows therefore the properties of our own preparations of heart sarcosomes are compared with those of liver mitochondria. Concerning liver mitochondria, we are essentially in agreement with other workers.

(1) *Respiratory control with isolated mitochondria.* Fig. 8 demonstrates a stimulation of 4·8-fold in the rate of oxidation of glutamate by heart sarcosomes, brought about by the addition of ADP. This is somewhat lower than the degree of stimulation which we obtained under similar conditions with liver mitochondria (about sevenfold); Lardy & Wellman (1952) found a stimulation of about tenfold, and Chance & Williams (1955*a*), under rather different conditions, report an even greater stimulation. The greater ATPase activity of heart sarcosomes in the presence of Mg^{2+} ions and absence of DNP, compared with liver mitochondria, might lead one to expect an even bigger difference in the respiratory-control indices of the two types of mitochondria. That it is not larger can be partly explained by the higher respiratory activity of the heart sarcosomes. A similar respiratory-control index was obtained with our usual preparations of sarcosomes, and with those preparations in which an attempt was made to keep the ATPase activity in the absence of DNP as low as possible.

(2) *Diphosphopyridine nucleotide content of isolated mitochondria.* Chance's (1956) statement concerning the low DPN content of heart sarcosomes is not supported by any of the published data on the sarcosomes. The high activity of the α-oxoglutaric oxidase system in the sarcosomes strongly suggested that they contained DPN, and this is confirmed by the direct analyses reported by Holton (1955) and in this paper. In fact, the sarcosomes contain somewhat more DPN than liver mitochondria. Although

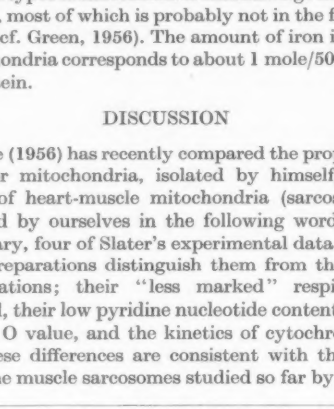

Fig. 10. Difference spectrum (anaerobic *minus* aerobic steady states) of sarcosome suspensions reduced by DL-β-hydroxybutyrate. ●, Rabbit-heart sarcosomes, 9·2 mg. of protein/ml.; DL-β-hydroxybutyrate, 0·0025M; ATP, 0·005M. Band widths, 4·3–4·7 mμ, expts. 56/35. ○, Rat-heart sarcosomes, 8·9 mg. of protein/ml.; DL-β-hydroxybutyrate, 0·0065M; ATP, 0·006M; sucrose, 0·28M; EDTA, 0·001M. Band widths, 4·3–4·5 mμ, expts. 56/36.

Table 7. *Calcium and iron content of rat-liver mitochondria and heart sarcosomes*

	Liver mitochondria	Heart sarcosomes
Calcium (μmoles/g. of protein)	61	99*
Iron (μmoles/g. of protein)	22	18

* From Slater & Cleland (1953).

Chance (1956) specifically refers to the 'low pyridine nucleotide content' of heart sarcosomes, it is possible that he may have had in mind enzymically reducible DPN (see Chance & Williams, 1956), and his comment was probably inspired by Holton's (1955) failure to demonstrate in the difference spectrum of sarcosomes a maximum which could be ascribed to reactions of endogenous DPN. It now appears that this failure was due to the restricted range of wavelengths studied. In work reported above, measurements extending between 300 and 445 mμ have detected a marked peak at 320 mμ in the difference spectrum of sarcosomes reduced by β-hydroxybutyrate. If the whole of the extinction at 320 mμ is assumed to result from reduction of bound DPN, its magnitude corresponds to between 55 and 80 % of that calculated from the measured DPN content of the sarcosomes. This calculation assumes that the extinction coefficient at 320 mμ of sarcosome-bound DPNH in a turbid suspension is equal to that of DPNH at 340 mμ in clear solution. In view of this and other uncertainties of the calculation (for instance, sarcosome-bound DPNH may be expected not to be fully oxidized in the aerobic steady state), these spectrophotometric results represent satisfactory agreement with the direct analyses. It is clear, then, that the DPN previously found by Holton (1955) in the sarcosomes is reducible by the β-hydroxybutyric dehydrogenase in these granules.

(3) *The low* P:O *ratio in heart sarcosomes.* The P:O ratios obtained with our sarcosomes are lower than those found with liver mitochondria, particularly with succinate as substrate.

In our attempts to determine the P:O ratio of heart sarcosomes (Slater & Holton, 1954), we were conscious of the fact that it would be difficult to isolate the sarcosomes from the muscle, where they are trapped between the myofibrils, without damaging some. There was a strong possibility therefore that our preparation would contain some damaged sarcosomes, even if most were intact. We therefore studied, in some detail, the effect of damaging sarcosomes in various ways: e.g. by mechanical damage, by swelling in hypotonic media, or by incubation (Slater & Cleland, 1953). The significant result was that while all these treatments of heart sarcosomes drastically reduced the activity of the α-oxoglutaric oxidase system, they had only a slight effect on the P:O ratio. Thus we felt confident that the damaged sarcosomes, which were probably present, would contribute little to the oxygen uptake with α-oxoglutarate as substrate, and since this oxygen uptake would be accompanied by only a slightly lower phosphorylation ratio, the final effect of the damaged sarcosomes on the measured phosphorylation ratio would be insignificant. Thus the measured ratio would represent the true ratio of the undamaged sarcosomes.

It must be emphasized that this argument was applied only to the heart sarcosomes oxidizing α-oxoglutarate. Our results indicated that the P:O ratio for the step α-oxoglutarate→succinate was very close to 3·0. This is not very different from liver mitochondria, and is not inconsistent with the generally accepted view that there are four phosphorylation steps in the α-oxoglutaric oxidase system (see Slater, 1956). This argument was not applied to the blowfly thoracic-muscle sarcosomes, since the P:O ratio was found very susceptible to various treatments (Lewis & Slater, 1954).

The argument was also not applied to the heart sarcosomes oxidizing succinate, since damage to the particles increased the respiratory activity (by decreasing the steady-state concentration of the inhibitory oxaloacetate) with a marked loss of phosphorylative activity. Thus the presence of damaged sarcosomes will appreciably lower the experimental P:O ratio when succinate is the substrate. It is significant that, in contrast with the very reproducible results obtained with α-oxoglutarate (Slater & Holton, 1954), our P:O ratios with succinate and rat-heart sarcosomes vary over a rather wide range, between 0·7 and 1·4 in sixteen experiments, with most values near 1. It is impossible for us to say whether our generally low values represent a difference between intact sarcosomes and liver mitochondria, or are due to damaged sarcosomes in our preparation. However, if the latter is the case it does mean that our sarcosomal preparation contains a large proportion of damaged sarcosomes (a mixture of 33 % damaged particles with zero phosphorylation and double the succinic oxidase activity of intact particles, and 66 % intact sarcosomes with a P:O ratio of 2 is necessary to give an experimental ratio of 1). Despite exhaustive attempts, we have not succeeded in isolating rat-heart sarcosomes which consistently have a P:O ratio much above 1 with succinate. We are continuing experiments in an attempt to explain the difference between our results and those of Maley & Plaut (1953, 1954) in this respect. Guinea-pig-heart sarcosomes often have higher ratios (K. Minnaert, unpublished work).

In our previous paper (Slater & Holton, 1954), we expressed the view that Copenhaver & Lardy's (1952) procedure, involving a short equilibration time and relatively small oxygen uptakes, appreciably underestimated the oxygen uptakes, and that consequently their values for the P:O ratios were too high. Although this was certainly the case when we used what we understood to be Copenhaver & Lardy's procedure, it now seems clear that no serious error accompanied their use of this technique (Lardy & Copenhaver, 1954). Our own

measurements shown in Table 1 do not, however, show any appreciable difference between the P:O ratios of heart and liver mitochondria oxidizing α-oxoglutarate, although our ratios with liver mitochondria oxidizing succinate and β-hydroxybutyrate are a little higher than those of Copenhaver & Lardy (1952).

(4) *Kinetics of cytochrome* b. There is a remarkable difference between the kinetics of cytochrome *b* in the two preparations. Holton (1955) has shown that if an anaerobic suspension of heart sarcosomes in a medium containing succinate and ADP is aerated, the cytochrome *b* is only partially oxidized (it remained nearly 100 % reduced in one experiment), but becomes more oxidized during the subsequent aerobic steady state. In contrast, cytochromes *c* and a_3 are immediately and completely oxidized, and remain oxidized during the aerobic steady state. The corresponding experiment has not been done with liver mitochondria, but Chance & Williams's (1955*a*) data suggest that under these conditions cytochrome *c* would be 13 % reduced, and cytochrome *b* 16 % reduced. The degree of reduction of cytochrome *b* in the aerobic steady state in the presence of ADP is then a clear-cut difference between the mitochondria isolated from the two tissues. It is important to note that the high degree of reduction of cytochrome *b* in the aerobic steady state is characteristic of the phosphorylating heart sarcosomes. The addition of DNP caused the cytochrome *b* to be largely oxidized in the steady state.

The explanation of the subsequent change in the steady state of cytochrome *b* has not been established. It is possible, however, that it coincides with the phosphorylation of the ADP to ATP. If this is the case, then it follows that as ADP is phosphorylated to ATP, the cytochrome *b* becomes more oxidized. This is the exact opposite of the behaviour of cytochrome *b* in liver mitochondria (Chance & Williams, 1955*a*).

Chance & Williams (1955*a*) have found an important difference between the kinetics of cytochrome *b* in phosphorylating mitochondrial preparations, where it is rapidly reduced, and in non-phosphorylating sarcosomal fragments (Keilin & Hartree heart-muscle preparation) where it is slowly reduced (Slater, 1950; Chance, 1952). Since the exact role of cytochrome *b* in the respiratory chain is still obscure, the significance of this difference in its kinetics remains uncertain. However, the high degree of cytochrome *b* in the aerobic steady state found by Holton (1955) in heart sarcosomes suggests that the rate of reduction of this cytochrome is not impaired in these granules.

(5) '*Direct evidence of damage*'. Since Lardy's (1956) paper refers to skeletal muscle, we cannot agree with Chance that it provides direct evidence of damage to our heart preparations. Skeletal muscle is intermittent in its activity, compared with the almost constant activity of the intact heart. Melrose, Dreyer, Bentall & Baker (1955) have reported experiments which provide good evidence of metabolic control in the intact heart (see Slater, 1957). However, it is not certain whether the control of the metabolic rate by muscular activity in heart and skeletal muscle is due to respiratory control or to the effect of muscular activity on the rate of glycolysis (Cori, 1956). If the latter is blocked, no substrate is supplied to the respiratory enzymes. In any case, the experiment described in Fig. 8 shows that isolated sarcosomes are capable of a considerable degree of respiratory control.

ATPase activity of liver and heart mitochondria

The enzyme systems catalysing the hydrolysis of ATP in the two types of mitochondria are very similar. Under the same conditions, the pH-activity curves were of the same general shape, although the positions of all the optima were about 0·2–0·3 pH unit lower in the sarcosomes than in the liver.

With regard to the question whether or not the isolated sarcosomes are damaged, the most interesting difference between the two types of mitochondria is the stimulation of the ATPase activity by added Mg^{2+} ions. As a consequence, the degree of stimulation by DNP, in the presence of magnesium at pH 7·4, was only about twofold with sarcosomes prepared in the way used in previous studies, compared with at least tenfold with liver mitochondria. This difference could be interpreted to support the contention that the sarcosomes are damaged. It is indeed probable that the sarcosomes isolated by the method of Cleland & Slater (1953) are slightly damaged since the ATPase activity in the absence of DNP can be lowered to some extent by varying the isolation procedure. However, we have not been able to isolate sarcosomes which show more than a fourfold increase in the latent ATPase activity at pH 7·4 on addition of 10^{-4} M-DNP to a reaction medium containing Mg^{2+} ions. Similar results were obtained by Chappell & Perry (1954) with pigeon-breast-muscle sarcosomes. The procedure used in the isolation of the sarcosomes from the hearts of young rats and guinea pigs was relatively mild and would not be expected to damage the sarcosomes more than the liver mitochondria would be damaged during their isolation.

It is possible that the moderately high ATPase activity observed in the absence of DNP is an intrinsic property of these sarcosomes. Perry (1956) has suggested that the sarcosomes isolated from pigeon-breast muscle contain a contractile system similar to actomyosin in that it requires ATP to function normally. It is possible that this contractile system is responsible for a portion

of the ATPase activity observed in the absence of DNP.

Perry's (1956) studies may also offer an explanation for the effect of including ATP in the isolation medium, since it is likely that the contracted sarcosomes would be more resistant to damage during the isolation procedure. The denser sarcosomes which are precipitated from the homogenate by centrifuging at low speeds probably represent those sarcosomes in which the most water has been squeezed out by the process of contraction.

Differences between liver and heart mitochondria

Both types of mitochondria exhibit a considerable degree of respiratory control with glutamate and a high phosphorylative activity with α-oxoglutarate. When fully activated by DNP, or by freezing and thawing, the pH–activity curves for the hydrolysis of ATP are very similar. There are, however, a number of differences between the two preparations, the most important of which are:

(1) The higher respiratory activity of the heart preparations, which is probably a consequence of their greater content of cytochromes (this is obvious to the eye, since liver mitochondria appear much paler than the heart preparations) and of DPN, and which may also be related to their greater ATPase activity after complete activation by ageing, freezing and thawing.

(2) The kinetics of cytochrome *b* considered above.

(3) The lower P:O ratio obtained with sarcosomes oxidizing succinate.

(4) The higher ATPase activity of sarcosomes in the presence of Mg^{2+} ions and absence of DNP.

Tapley & Cooper (1956) have shown that heart sarcosomes are much more resistant than liver mitochondria to swelling induced by thyroxine. There is also evidence from the effect on the P:O ratio with ferrocytochrome *c* as substrate that heart sarcosomes are more resistant than liver mitochondria to morphological changes induced by hypotonicity (Slater, 1956).

Less important differences are the low myokinase (Cleland & Slater, 1953) and rhodanese (Moyle, 1953) activity of heart sarcosomes.

It seems likely that the high respiratory activity of isolated heart sarcosomes is a physiological characteristic, and is not brought about during their isolation. Although it is not possible at present to say that this is also the case with the other differences between liver and heart mitochondria, the work reported above can be as well explained in terms of physiological as of artifactual contrasts. It would thus seem preferable for calculations relating to muscle physiology to be based on biochemical properties of muscle sarcosomes, as these become better known, rather than on the properties of liver mitochondria.

SUMMARY

1. Succinate and α-oxoglutarate were oxidized faster by rat-heart sarcosomes (mitochondria) than by rat-liver mitochondria. β-Hydroxybutyrate was oxidized at about the same rate by the two preparations.

2. Heart sarcosomes oxidizing succinate and β-hydroxybutyrate yielded lower P:O ratios than liver mitochondria oxidizing the same substrates. The maximum rate of phosphorylation per unit weight (Q_P) was greater in heart sarcosomes than in liver mitochondria.

3. The properties of the enzyme systems in the two types of mitochondria after activation by the addition of dinitrophenol or by freezing and thawing, bringing about the hydrolysis of added adenosine triphosphate (ATP), were very similar. In the absence of dinitrophenol or other activating treatment, magnesium stimulated these enzyme systems in heart sarcosomes, but had little effect on the hydrolysis of ATP by liver mitochondria.

4. Factors affecting the degree of respiratory control in the two types of mitochondria are similar. The addition of adenosine diphosphate to heart sarcosomes oxidizing glutamate stimulated the rate of respiration about fivefold. The greater degree of respiratory control in liver mitochondria may be satisfactorily explained by their lower ATPase activity.

5. Both spectrophotometric measurements of endogenous pigments in the intact mitochondria and direct chemical analyses showed that liver and heart mitochondria contained similar amounts of diphosphopyridine nucleotide. They also contain similar amounts of iron. Heart sarcosomes contain a considerably higher concentration of the cytochrome system and a greater specific ATPase activity after full activation.

6. The differences between the enzymic behaviour of heart sarcosomes and liver mitochondria are discussed. The higher respiratory activity of isolated sarcosomes is probably a physiological characteristic, and is not brought about during their isolation. Although it is not possible at present to be certain that this is also the case with the other differences between liver and heart mitochondria, the work reported in this paper is more readily explicable in terms of physiological rather than artifactual contrasts, and offers no support for the idea that their patterns of enzymic behaviour are identical *in vivo*.

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Purine and Pyrimidine Transaminases in *Escherichia coli**

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Biological conversion of amino purines and pyrimidines into their hydroxy compounds has hitherto been ascribed exclusively to the action of hydrolytic deaminases. The report that suspensions or extracts of *Escherichia coli* (strains B and Crookes) catalyse transamination between α-oxoglutarate and adenine, guanine or cytosine (Gunsalus & Tonzetich, 1952) would point to the existence of important alternate metabolic pathways for these constituents of nucleic acids. The transamination reaction, as usually understood, involves an intermolecular oxidation–reduction. This reaction is not possible with an intact purine or pyrimidine ring; therefore transamination of these compounds must involve a mechanism not previously described or must be preceded by a change in the ring such as reduction. It seemed therefore of importance to repeat and to extend the experiments of Gunsalus & Tonzetich (1952). We have not been able to confirm their observations by employing similar bacteria and similar experimental conditions. Nor was transamination evident, when, in a limited number of experiments, mammalian-liver homogenates or slices replaced bacterial preparations.

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MATERIALS AND METHODS

Chemicals. The chemicals used were obtained as follows: adenosine and cytidine (ribosides), cytosine and ninhydrin, from Nutritional Biochemicals Corp., Cleveland, Ohio, U.S.A.; adenine and glutamic acid, CfP brand, from the California Foundation for Biochemical Research; reduced diphosphopyridine nucleotide (DPNH) from Sigma Chemical Co., St Louis, Mo., U.S.A.; α-oxoglutaric acid, lot IV-8A from the California Foundation for Biochemical Research; pyridoxal phosphate (Ca salt, 100 mg. = approx. 70 mg. of pyridoxal) from the Merck Institute for Therapeutic Research.

Bacterial suspensions and extracts. A loop of growth from a slant of *E. coli* (strains B and Crookes) was transferred to fresh nutrient agar and incubated at 37° for 18–24 hr. Loops of the actively growing bacteria were used to seed 500 ml. of broth having the same composition as the subsequent medium. In 24 hr. dense growth of bacteria resulted and all of the culture was used to inoculate 10–20 l. of medium. The composition of the latter medium and the growth and harvesting of cells were as described by Gunsalus & Hand (1941). The final bacterial suspensions were freeze-dried and stored at −15°. Several dried batches of the same strain of bacteria were combined. Just before use, weighed amounts of the dried cells were suspended in water or in phosphate buffer and used without further treatment. Cell-free extracts were occasionally prepared by the method of Gunsalus & Tonzetich (1952).

From a slant of *E. coli*, American Type Culture (A.T.C.) no. 4157, cells were grown and harvested as described by Cohen (personal communication). These cells were freeze-dried and stored at −15°. Suspensions of A.T.C. no. 4157 in acetate buffer pH 5 (prepared just before use) were used as a source of glutamic acid decarboxylase.

Liver homogenates and slices. Homogenates of rabbit liver (1 vol. of liver and 4 vol. of either water or phosphate buffer) were prepared according to the method of Potter & Elvehjem (1936).

Slices (approx. 0·5 mm. thick and weighing 50–75 mg.) were cut from rat liver with a Stadie–Riggs microtome. All the operations with fresh tissue were carried out in a cold room at 5–6°. Approx. 30 min. elapsed between killing the animal and starting the reaction by adding homogenate or slices to the medium.

Conditions for measuring transamination

In all four methods which follow, the α-oxoglutarate and the amino compounds were dissolved in 0·05 M-phosphate buffer, pH 7·4. All phosphate buffers were made by mixing KH_2PO_4 and Na_2HPO_4.

Method 1. The substrate control consisted of the following: 0·5 ml. of pyridoxal phosphate (0·06 μmole), 1 ml. of 0·05 M-phosphate buffer, pH 7·4, 0·5 ml. of α-oxoglutarate (40–80 μmoles), and 1 ml. of amino compound (25 μmoles of adenine, cytosine, adenosine or cytidine). For the enzyme control, the amino compound was replaced by 1 ml. of enzyme preparation. For the complete system, the phosphate buffer was replaced by 1 ml. of enzyme preparation. The enzyme preparation was 20% (v/v) of rabbit-liver homogenate in 0·05 M-phosphate buffer, pH 7·4.

The reaction was carried out in 15 ml. screw-topped culture tubes which were continuously shaken at 37°. After 1 hr., 7 ml. of ethanol was added to each tube and the insoluble material centrifuged off. The supernatant fluid was evaporated to dryness. The dry residue was dissolved in 1 ml. of water. Samples were spotted on Whatman filter paper no. 1. Phenol–water (80:20, w/v) mixture was employed as the solvent for descending chromatography. The dried paper was treated with ninhydrin. The amount of glutamate formed was based on visual comparison with known amounts of glutamic acid (0·013–0·156 μmole) simultaneously spotted and run together with the unknowns.

Method 2. This was essentially the method of Gunsalus & Tonzetich (1952). The composition of the solution for the substrate control was as follows: 0·3 ml. of pyridoxal phosphate (0·04 μmole), 0·1 ml. of M-phosphate buffer, pH 8·3, 0·4 ml. of α-oxoglutarate (25 μmoles) plus amino compound (32 μmoles), and 0·2 ml. of water. For the enzyme control, the amino compound was omitted and the water replaced by 0·2 ml. of a bacterial extract or suspension (15 mg. dry wt. of bacteria) in 0·1 M-phosphate buffer, pH 8·3. For the complete system the water was replaced by 0·2 ml. of the bacterial extract or suspension.

Conditions for the reaction were as described for Method 1. The reaction was stopped by the addition of 0·1 ml. of melted trichloroacetic acid. After centrifuging, 50 μl. portions of the clear supernatant were spotted on paper and all subsequent steps were carried out as in method 1. Glutamic acid standards were made up in trichloroacetic acid solution.

Method 3. The composition of the test solution was essentially the same as in method 2 except that 0·2 ml. of DPNH (5 μmoles) was added to all reaction tubes and the bacterial suspensions or extracts were made up in 0·1 M-phosphate buffer, pH 7·4. The reaction was stopped by the addition of 1 ml. of ethanol. The clear supernatant fluid produced by centrifuging was used for spotting and the subsequent steps in the development of the chromatogram were the same as in method 1.

Elution of the ninhydrin-coloured areas with an R_F corresponding to glutamate and spectrophotometric determination of glutamate were carried out essentially by the method of Giri, Radhakrishnan & Vaidyanathan (1952).

Method 4 (manometric method). The substrate control contained the following: 0·3 ml. of pyridoxal phosphate (0·12 μmole), 1 ml. of 0·1 M-phosphate buffer, pH 8·3, 0·5 ml. of α-oxoglutarate (40 μmoles), 1 ml. of amino compound (25 μmoles). The enzyme control contained 1 ml. of bacterial preparation or liver homogenate in place of the buffer, and the amino compound was replaced by 1 ml. of 0·05 M-phosphate buffer, pH 7·4. For the complete system the pH 8·3 buffer was replaced by 1 ml. of enzyme solution. The amino compound was adenine, cytosine, adenosine or cytidine.

The bacterial enzyme preparations used were suspensions or extracts of *E. coli* (strains B or Crookes). Suspensions contained 15 mg. of dried cells; extracts were derived from 24 mg. of cells. Both were made up in 0·1 M-phosphate buffer, pH 8·3. The enzymic preparation from rabbit liver was a 20% (v/v) homogenate in 0·1 M-phosphate buffer, pH 8·3.

The reaction was carried out in Warburg flasks at 37° for 1 or 2 hr. The temperature of the flasks was then rapidly brought to 100° and maintained at this level for 10 min. to stop the reaction. The contents were cooled and then brought to pH 5 by the addition of 0·5 ml. of 3 M-acetate buffer,

pH 4·8, and glutamate was determined according to a modification of the usual manometric procedure (Gale, 1945; Krebs, 1948), whereby *E. coli* A.T.C. no. 4157 is employed (Dr P. Cohen, personal communication) instead of *Clostridium welchii* as a source of glutamic acid decarboxylase.

Recovery of formed glutamate was estimated by replacing the amino compounds in some flasks with known amounts (3–8 μmoles) of glutamate; evolution and measurement of CO_2 derived from glutamate also served to test the activity of the glutamic acid decarboxylase.

EXPERIMENTAL AND RESULTS

It was impossible to prepare a solution containing 25 μmoles of guanine/ml. at pH 8·3, as specified by Gunsalus & Tonzetich (1952). Aqueous solutions of guanine at 40° contain about 0·26 μmole/ml. (Wood, 1903). A concentration of not more than 6 μmoles of guanine/ml. at pH 8 could be obtained by resorting to colloidal suspensions containing gelatin (Schmidt, 1932). The uncertainty of the actual guanine concentration in such colloidal solutions prompted us to omit guanine from our experiments.

Table 1 lists the results obtained in experiments similar to but not identical with those of Gunsalus & Tonzetich (1952). It may be observed that little or no formation of glutamate could be detected on chromatograms when adenine, cytosine, adenosine or cytidine, together with α-oxoglutarate, were incubated with bacterial preparations, rabbit-liver homogenates or rat-liver slices. Thus evidence for transamination involving adenine, cytosine, their respective nucleosides and α-oxoglutarate in solutions containing enzyme preparations was not obtained.

Manometric determinations of glutamate, with a preparation of *E. coli*, A.T.C. no. 4157, as a source of glutamic acid decarboxylase, confirmed the negative results of chromatography for rabbit-liver homogenate and *E. coli* B.

The chromatographic methods for measuring glutamic acid were tested by replacing the amino compound with glutamic acid in solutions which contained enzyme preparations and in those which did not. The activity of A.T.C. no. 4157 suspensions was checked in each experiment with known amounts of glutamate.

Recoveries of added glutamate in various experiments ranged from 63 to 125 % from the chromatographic experiments and from 73 to 110 % for the manometric experiments (Table 2).

Not included in Table 2 are the results of a large series of experiments in which 25 μmoles of glutamate were added to solutions which contained suspensions or extracts of both *E. coli* B and Crookes, and to solutions which were enzyme-free. Recoveries of glutamate by chromatography (Method 3) in all of these experiments were in the range 88–116 %.

However, the distinct possibility remained that small amounts of glutamate could actually have been formed, and have remained undetected either because of analytical limitations or because the amino acid was itself metabolized by the bacteria. To test this possibility, 4 or 8 μmoles of glutamate were incubated with *E. coli* Crookes as described in method 3. It may be seen (bottom of Table 2) that whereas approx. 2·5 μmoles or 63 % of glutamate were recovered when 4 μmoles were incubated with *E. coli* Crookes the corresponding average recovery was 7·1 μmoles or 89 % when 8 μmoles were incubated. This is in contrast to recoveries of 90–98 % and 100–106 % in similar experiments in which bacterial preparations were not added to the media. The higher recoveries obtained when larger amounts of glutamate are employed may tend to obscure small but fairly constant losses. These losses in the

Table 1. *Glutamate formation from adenine, cytosine and their nucleosides*

All listed results are the average of duplicate complete runs. The last two expts. were done manometrically; the remainder were done chromatographically. For conditions see Methods.

Enzyme source	Method no.	Glutamate formed (μmoles)				
		Enzyme blank	+Adenine	+Cytosine	+Adenosine	+Cytidine
Rabbit-liver homogenate	1	No discernible increase of area or intensity of coloured spots over those of enzyme controls				
E. coli B, extract	2					
E. coli Crookes, extract	2					
E. coli B, suspension	3	Trace	Trace	Trace	—	—
E. coli B, suspension	3	1·3	1·3	1·0	0·2	0·6
E. coli B, extract	3	0·6	0·7	1·6	0·2	0·3
E. coli Crookes, suspension	3	Trace	Trace	Trace	—	—
E. coli Crookes, suspension	3	0·7	0·7	0·6	—	—
E. coli Crookes, extract	3	0·8	0·8	0·9	—	—
Rat-liver slices	3	2·7	1·7	1·6	1·2	0·9
Rabbit-liver homogenate	4	1·9	2·5	2·7	2·5	2·7
E. coli B	4	Trace	Trace	Trace	Trace	Trace

Table 2. *Recovery of added glutamate*

All listed results represent the average of at least duplicate complete experiments. For conditions see Methods.

Enzyme source	Glutamate		
	Added (μmoles)	Recovered (μmoles)	Recovered (%)
Manometric (Method no. 4)			
None	3·0	2·2	73
	3·0	2·5	83
	3·0	3·0	100
	4·0	4·0	100
	6·2	5·1	82
	6·2	5·2	84
	6·2	6·8	110
	8·0	7·2	90
Rabbit-liver homogenate	3·0	2·8	93
	3·0	2·6	87
	6·0	5·4	90
	6·4	5·4	84
Rabbit, boiled-liver homogenate	6·0	5·5	91
E. coli B, boiled-cell suspension	4·0	3·4	85
	8·0	6·8	85
Chromatographic (Method no. 3)			
E. coli B, suspension	25	27	108
	25	26	104
E. coli B, extract	25	25	100
E. coli Crookes, suspension	25	22	88
	25	26	104
E. coli Crookes, extract	25	29	116
Rat-liver slices	4·0	5·0	125
	8·0	7·2	90
None	4·0	3·6	90
	4·0	3·8	95
	4·0	3·9	98
E. coli Crookes, suspension	4·0	2·5	63
	4·0	2·5	63
	4·0	2·6	65
None	8·0	8·5	106
	8·0	8·1	101
	8·0	8·0	100
E. coli Crookes, suspension	8·0	7·4	93
	8·0	6·8	85
	8·0	7·1	89

presence of the bacterial extract or suspension, ranging from 0·6 to 1·5 μmoles, represent the maximum limiting amounts that could be formed by transamination and yet escape detection.

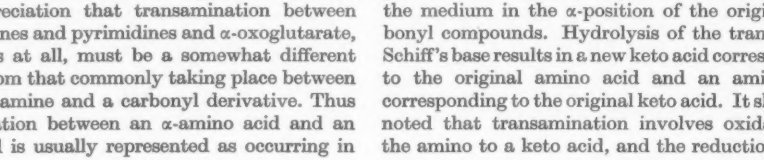
DISCUSSION

The failure to confirm the results of Gunsalus & Tonzetich (1952), who reported as much as 6 μmoles of glutamate formed by transamination between amino purines and amino pyrimidines and α-oxoglutarate, may stem from a variety of causes, among them differences in bacterial strains and composition of the culture media, growth and harvesting conditions, techniques of measuring glutamate, etc. We endeavoured to obtain *E. coli* strains B and the Crookes strain from the original Laboratory (Cornell University); however, our bacterial preparations were ultimately derived from slants kindly sent to us from Illinois by Dr I. C. Gunsalus. We have no certain knowledge that either our strains or our culture media were identical with those employed by Gunsalus & Tonzetich (1952). The procedures for growth and harvesting are fairly routine operations; minor variations are not usually considered capable of producing qualitative changes in bacterial-enzyme content.

Our later procedures for estimating glutamate were based on elution from paper and spectrophotometric determination of the chromogen produced by the reaction between glutamate and ninhydrin; also glutamate was quite independently and specifically estimated by a manometric technique. These methods are in contrast to visual comparison of glutamate on ninhydrin-treated paper chromatograms (Gunsalus & Tonzetich, 1952).

A theoretical difference in our approach lay in the early appreciation that transamination between amino purines and pyrimidines and α-oxoglutarate, if it occurs at all, must be a somewhat different reaction from that commonly taking place between a primary amine and a carbonyl derivative. Thus transamination between an α-amino acid and an α-keto acid is usually represented as occurring in a number of steps (Fig. 1, Scheme 1). A Schiff's base is formed by condensation of the amino and the keto acid. The α-hydrogen (proton) of the amino donor becomes labile in the presence of the keto acid (Braunstein, 1947). Actually this hydrogen may dissociate even under conditions excluding any transamination (Sprinson & Rittenberg, 1951). Once the Schiff's base is formed, however, both dissociation of the α-proton and an electromeric shift in the methylene–azomethine bridge take place. Simultaneously, a proton is taken up from the medium in the α-position of the original carbonyl compounds. Hydrolysis of the transformed Schiff's base results in a new keto acid corresponding to the original amino acid and an amino acid corresponding to the original keto acid. It should be noted that transamination involves oxidation of the amino to a keto acid, and the reduction of the

Scheme 1



Scheme 2

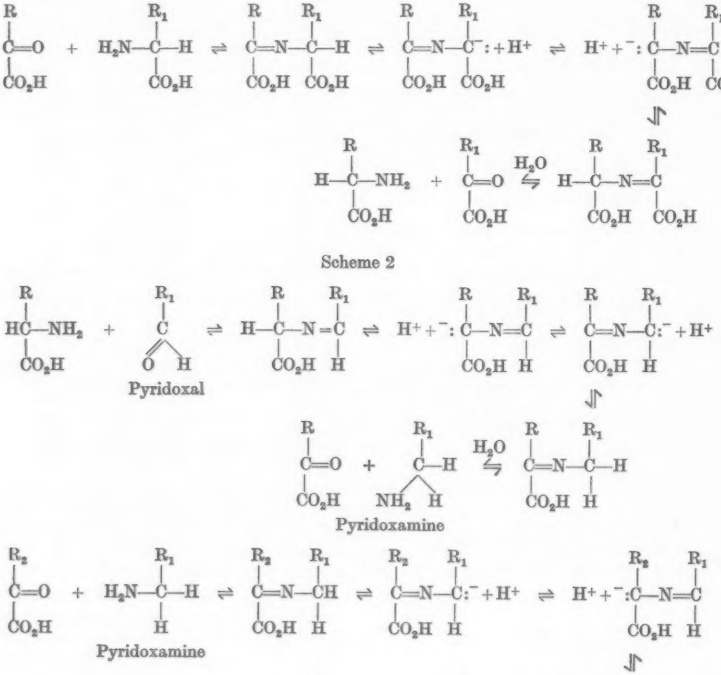


Fig. 1. Reaction schemes for transamination.

keto to an amino acid. The intramolecular oxido-reductions or reversible prototropic change in the methylene–azomethine bridge (interconversion of the two Schiff's bases) is thought to be the enzyme-catalysed step. In his review, Braunstein (1947) states: 'It appears that dissociation of an alpha hydrogen is an independent, relatively non-specific and heat-resistant function of aminopherase, prerequisite for...transfer of amino groups (and electrons), i.e. by transamination proper.'

More elaborate schemes involving pyridoxal–pyridoxamine (Meister, 1955; Tannenbaum, 1956), while undoubtedly representing a more valid picture of what actually happens, have not contributed a clearer understanding of the fundamental mechanism of transamination. Examination of such a scheme (Fig. 1, Scheme 2) in which pyridoxal–pyridoxamine is included reveals that the original amino and keto acids do not directly interact, but that the amino group is transferred via the pyridoxal–pyridoxamine system again through Schiff's base-type compounds. Implicit, also, is the labilization of a proton of the amino methyl group of pyridoxamine in the usual fashion during transamination.

With adenine or cytosine, neither of the tautomeric structures (containing an amino or imino group respectively) has a proton at C-6. Thus an intramolecular oxidation–reduction cannot take place in these compounds similar to that occurring when amino acids act as amino donors in transamination. Dr A. Meister (personal correspondence) was kind enough to call our attention to a recent report by Mardashev & Pavlova (1955), describing transamination between amino purines and glyoxylic or glycollic acids. It would be difficult to reconcile such transfers of amino groups with present mechanisms proposed for transamination.

The tautomeric form which contains an imino group at C-6 is presumably not capable of undergoing transamination. Inspection of a current list of compounds which are reputed to engage in transamination fails to reveal the presence of an imino compound (Meister, 1955).

It would seem therefore that a prior reduction or opening of the pyrimidine ring is an obligatory prerequisite for transamination in adenine and cytosine. For this reason we included a common biological reducing agent, DPNH, in many of our test solutions. There was no evidence that such inclusion altered any experimental results.

No attempt was made to follow intrinsic purine or pyrimidine changes. It was felt that interpretation of such data would be complex since specific purine and pyrimidine deaminases and nucleoside deaminases are usually active in bacteria (Stephenson & Trim, 1938).

The results are fairly conclusive in that no transamination could be demonstrated between adenine, cytosine, adenosine or cytidine and α-oxoglutarate when *E. coli* strain B, *E. coli* strain Crookes, rabbit-liver homogenate or rat-liver slices were used as possible enzyme sources. However, with the bacteria small amounts of glutamic acid (approx. 1 μmole) could have been formed by transamination and not have been measured. This is the amount of glutamate that could not be accounted for in recovery experiments when 4 or 8 μmoles of glutamate were incubated with *E. coli*, Crookes strain. Isotopically labelled compounds could conceivably be of aid in resolving the question whether transamination occurs to a very slight extent. We intend to investigate this possibility in the future.

Compounds other than α-amino and keto acids are known to participate in biological-transamination reactions. Indeed the basic requirements are reputed to be an unsubstituted amine, a carbonyl group, enzyme and pyridoxal or pyridoxamine. Another requirement, not specifically mentioned but suggested by examination of the list of compounds which may participate in transamination (Meister, 1955), is a proton on the carbon linked to the free amino group.

SUMMARY

1. No transamination could be demonstrated between α-oxoglutarate and adenine, adenosine, cytosine or cytidine, with *Escherichia coli* (strains B and Crookes), rabbit-liver homogenate and rat-liver slices as enzyme sources.

2. Analytical limitations and the possibility of slight metabolic utilization of formed glutamate by the bacteria renders equivocal the conclusion that transamination of adenine, cytosine and their ribosides is altogether absent, but the existence of a transamination reaction for these compounds must be sought by other techniques.

Pyridoxal phosphate was a gift from Dr W. Umbreit of the Merck Institute for Therapeutic Research.

We are indebted to Dr I. C. Gunsalus for sending us slants of *E. coli* (strains B and Crookes).

We are indebted to Dr Philip Cohen for sending us a flow sheet for the preparation of A.T.C. no. 4157. We also followed his suggestions for the manometric determination of glutamate. We also wish to thank Mrs S. Zehl who carried out some of the analyses for glutamate.

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The Hydrolysis of Indoxyl Esters by Rat Esterases

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It has been reported previously that human blood contains five esterases capable of hydrolysing unsubstituted indoxyl esters (Underhay, 1957). Three of these esterases, acetocholinesterase, an aliphatic esterase and an aromatic esterase, are found in the erythrocytes and the remaining two esterases, butyrocholinesterase and an aromatic esterase, which is not identical with the aromatic esterase of the erythrocytes, are present in plasma. When either human erythrocytes or human plasma are used as enzyme source, hydrolysis is mainly accomplished by the cholinesterases and the other esterases participate only to a small extent. In this paper, for reasons to be discussed below, the term 'aliphatic esterase' is replaced by 'organophosphate-sensitive esterase' and the term 'aromatic esterase' by 'organophosphate-resistant esterase'.

Most of the histochemical studies in which indoxyl esters have been used as substrates have so far been carried out on rat tissues (Holt, 1954, 1956). Experiments were therefore carried out to study the hydrolysis of unsubstituted indoxyl esters by erythrocyte preparations and plasma of the rat and by homogenates of rat liver from which the nuclei had been removed. The results obtained show that the hydrolysis of these esters by the three rat-enzyme preparations is mainly accomplished by organophosphate-sensitive esterases and that the cholinesterases and organophosphate-resistant esterases contribute to a lesser extent.

MATERIALS AND METHODS

Substrates. The substrates used were acetylcholine chloride, propionyl- and butyryl-choline iodides, indoxyl acetate, propionate and butyrate and α-naphthyl acetate. All were of analytical purity. Acetylcholine chloride was purchased from Roche Products Ltd. and the other substrates were kindly provided by Dr S. J. Holt.

Inhibitors. Inhibitors used were eserine sulphate, 3-(diethoxyphosphinyloxy)-*N*-methylquinolinium methyl sulphate (Ro 3–0422), diethyl *p*-nitrophenyl phosphate (E 600), di*iso*propyl *p*-nitrophenyl phosphate (DINP), di*iso*propyl phosphorofluoridate (DFP) and the selective anti-acetocholinesterase 3-oxo-1:5-diphenylpentane-*pp'*-bis(propyldimethylammonium) dibromide (B.W. 285C51).

Enzyme preparations. Male white Wistar rats were used for all experiments. Blood was rapidly withdrawn by cardiac puncture under ether anaesthesia and collected in a tube containing heparin. The plasma was pipetted off after the blood had been centrifuged for 10 min. at 3700 rev./min. in an International Centrifuge, size 1, Type SB, and the erythrocytes were twice washed with 0·9% NaCl. Since the erythrocytes agglutinate rapidly on standing they were immediately haemolysed with 9 vol. of 0·025 M-$NaHCO_3$. Throughout the text the terms 'erythrocyte preparation' and 'plasma' refer to preparations obtained in this way. Zajicek & Datta (1952, 1953) and Hines (1952) have shown that platelets and white cells of rats also contain acetocholinesterase. To investigate the effect of these components on the activity of the enzyme preparations described above, the following experiments were carried out. A cell count of one of the plasma samples showed that the number of platelets and white cells present was negligible; hydrolysis resulting from this contamination is too small to be recorded under the experimental conditions and more than 99% of the hydrolysis obtained with plasma as enzyme source is due to enzymes present in the plasma itself.

A cell count of two samples taken from one of the erythrocyte pellets showed that although both white cells and platelets were present they were far more numerous in the sample taken from the top than in that taken from the bottom of the pellet. Table 1 gives the rates of hydrolysis of acetylcholine and indoxyl acetate by the two samples together with a count of the cell types present. Also included are the rates of hydrolysis of both substrates by two different preparations of platelets. These results show that the platelets contribute approximately 30% to the hydrolysis of acetylcholine when erythrocyte preparations are used as enzyme source. With indoxyl acetate as substrate their contribution does not amount to more than 10% of the total hydrolysis. Inhibition studies with a 10^{-7} M-concentra-

Table 1. *Effect of platelets and white cells on the rate of hydrolysis by erythrocyte preparations*

A. Two samples were withdrawn from different levels of the erythrocyte pellet, one from the bottom and the other from near the top. After diluting with normal saline, the enzymic activity of each was determined manometrically and the cell counts were carried out.

B. For the platelet preparations sodium citrate was used as anticoagulant. After centrifuging for 10 min. at 1500 rev./min. the platelets were obtained from the supernatant by centrifuging at 3000–4000 rev./min. for 15 min. The platelets were washed three times in normal saline solution and finally resuspended after breaking up the clumps. The error in the platelet count is estimated to be about ±20%.

	Cells/mm.³			Hydrolysis (μl. of CO_2/10 min./0·1 ml. of enzyme soln.)	
	Erythrocytes	Platelets	White cells	Acetylcholine	Indoxyl acetate
A. Top sample	16 000 000	1 000 000	16 000	18·5	65·8
Bottom sample	16 000 000	12 000	1 000	12·7	59·4
B. Platelet preparation 1	—	500 000	—	4·9	6·6
Platelet preparation 2	6 000	510 000	2 000	5·0	6·8

tion of Ro 3–0422 (see below) showed that 85% of the hydrolysis of indoxyl acetate by platelets is accomplished by cholinesterase. From the results in Table 1 no conclusions can be drawn about the enzymic activity of white cells but it seems that any hydrolysis accomplished by them is not measurable under the experimental conditions.

The method of de Duve, Pressman, Gianetto, Wattiaux & Appelmans (1955) was used to separate liver homogenates into two fractions, one of which contained the nuclei and the other the residual homogenate (fraction E). Both the nuclei and fraction E were made up as 10% (w/v) concentrates with 0·25 M-sucrose, and 0·025 M-$NaHCO_3$ was used for subsequent dilutions.

Measurement of esterase activity

Enzyme activities were determined manometrically in the Warburg apparatus at 37° in a medium of 0·025 M-$NaHCO_3$, containing 0·1% (w/v) of crystallized bovine-plasma albumin (Armour Laboratories) and saturated with N_2+CO_2 (19:1) (pH 7·5) as described by Underhay (1957).

Substrate (0·3 ml.) dissolved in 50% (v/v) ethanol was used in each manometric vessel and the final substrate concentration was 6 mM in each case. The concentration of ethanol in the reaction mixture never exceeded 5% (v/v) (see Underhay, 1957) and control experiments showed that such a concentration of ethanol had no effect on the hydrolysis of acetylcholine by any of the enzyme preparations used.

In experiments involving the use of an inhibitor, enzyme and inhibitor were kept in contact for 20 min. at 37° before the addition of the substrate. The expression I_{50} denotes the concentration of a substance which inhibits hydrolysis by 50%.

The activities of enzyme preparations obtained from different rats were variable, especially with plasma, and the results given in Tables 2, 3 and 5 are means of several determinations. In a previous publication (Underhay, 1957) esterases which are inhibited by organophosphates, but not by eserine, were referred to as aliphatic esterases and those which are inhibited neither by organophosphates nor by eserine as aromatic esterases. Since it is known that the substrate specificity of these enzymes is not necessarily in accordance with these names (Mounter & Whittaker, 1953; Aldridge, 1954), and since it has been found in the experiments described in this paper that the indoxyl esters, which are aromatic compounds, are very rapidly hydrolysed by an organophosphate-sensitive enzyme in rat liver, the term 'aliphatic esterase' is replaced by 'organophosphate-sensitive esterase' and the term 'aromatic esterase' by 'organophosphate-resistant esterase'. The former should not be confused with the cholinesterases, which are also organophosphate-sensitive; the cholinesterases form an entirely separate group and are distinguished from other esterases by their high sensitivity to eserine and to Ro 3–0422 (see below). These terms are preferred to the Aldridge nomenclature of 'A' and 'B' esterase since the organophosphate-sensitive esterase does not, in this case, hydrolyse butyrates at the highest rate.

RESULTS

Hydrolysis of choline and indoxyl esters by esterases of rat-erythrocyte preparations

The rates of hydrolysis of the three indoxyl esters by erythrocyte preparations were compared with those of the corresponding choline esters. The results in Table 2 show that all the indoxyl esters are hydrolysed more rapidly than any of the choline esters. With both types of ester, the rate of hydrolysis is inversely related to the length of the acyl chain.

To determine the extent to which the different esterases contribute to the hydrolysis of indoxyl esters the effect of various inhibitors was investigated.

Eserine. Eserine (10^{-5} M) inhibits hydrolysis of acetylcholine almost completely, but reduces hydrolysis of indoxyl acetate by only 25%. Similarly, inhibition of the hydrolysis of indoxyl propionate and butyrate by 10^{-5} M-eserine amounts to 45 and 8% respectively (Table 3). Similar results are obtained with 10^{-4} M-eserine. Under the experimental conditions cholinesterase activity is completely inhibited by B.W. 285C51 and thus the eserine-sensitive hydrolysis of the indoxyl esters and the hydrolysis of the choline esters are accomplished by an acetocholinesterase.

Ro 3–0422. This inhibitor is a far more potent anticholinesterase than eserine, and cholinesterase

Table 2. *Rate of hydrolysis by rat-erythrocyte preparations and plasma*

Esterase activity was determined manometrically at 37° with 0·025 M-$NaHCO_3$ as medium and a substrate concentration of 6 mM. Standard deviations are included and the number of determinations given in parentheses.

	Choline esters	Indoxyl esters	α-Naphthyl esters
	(μl. of CO_2/10 min./0·1 ml. of erythrocyte preparation)		
Acetate	15·6±2·5 (11)	60·7±5·1 (10)	57·4±11·4 (4)
Propionate	12·8±1·5 (2)	27·1±1·6 (4)	—
Butyrate	0·8±0·2 (3)	18·1±1·7 (4)	—
	(μl. of CO_2/10 min./0·2 ml. of plasma)		
Acetate	19·8±4·9 (11)	72·2±13·2 (10)	3660·0±61·6 (4)
Propionate	32·4±9·4 (2)	163·5±26·3 (7)	—
Butyrate	11·3±3·3 (8)	48·8±7·1 (4)	—

Table 3. *Inhibition of hydrolysis by erythrocyte preparations and plasma by eserine, Ro* 3–0422 *and diisopropyl phosphorofluoridate*

Enzyme and inhibitor were incubated together for 20 min. at 37° before the substrate was added from the side arm. Inhibition is expressed as a percentage of untreated controls. Standard deviations and the number of determinations (in parentheses) are given. A, Acetate; P, propionate; B, butyrate.

	Choline esters			Indoxyl esters			α-Naphthyl esters
Inhibitor	A	P	B	A	P	B	A
				Erythrocyte preparation			
10^{-5} M-Eserine	98	—	—	25±8·3 (7)	45±2·1 (3)	8±12·5 (3)	39±2·0 (4)
10^{-7} M-Ro 3–0422	100	100	—	27±6·8 (6)	47±5·0 (2)	6±8·6 (2)	41±1·6 (3)
10^{-5} M-DFP	100	—	—	43·5±13·7 (6)	83±2·0 (3)	98±1·4 (3)	77±5·5 (4)
				Plasma			
10^{-5} M-Eserine	95	—	92	28±6·4 (6)	7±9·7 (6)	0±3·9 (3)	7±7·9 (4)
10^{-8} M-Ro 3–0422	98	—	90	25±8·7 (7)	5±12·7 (5)	0±5·0 (2)	0±5·0 (2)
10^{-5} M-DFP	100	—	—	95±5·0 (3)	99±2·2 (2)	99±1·6 (2)	99±1·4 (2)

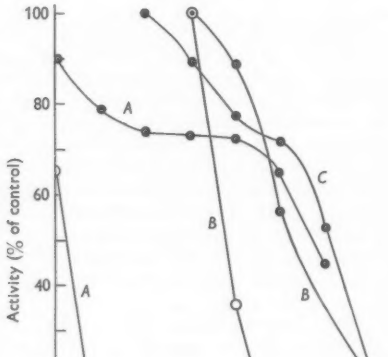

Fig. 1. Inhibition of hydrolysis by erythrocyte preparations after 20 min. pre-incubation of the enzyme with the inhibitor. Substrate concn. was 6 mM. ○, Acetylcholine; ●, indoxyl acetate. Curve *A*, Ro 3–0422; *B*, DFP; *C*, E 600.

inhibition obtained by this compound is not reversed by substrate (Hobbiger, 1954). From experiments in which various concentrations of Ro 3–0422 were used it can be calculated that the I_{50} for the acetocholinesterase of rat-erythrocyte preparations is $1·7 \times 10^{-10}$ M, and a 10^{-9} M-concentration inhibits the enzyme almost completely (Fig. 1).

If indoxyl acetate is used as substrate the inhibition curve shows two inflexions (Fig. 1). Twenty-seven per cent. of the hydrolysis is highly sensitive to Ro 3–0422 and the I_{50} of the enzyme involved is of the same order as that of acetocholinesterase. The inhibition curve shows a plateau between 10^{-8} and 10^{-6} M and the percentage inhibition achieved by these concentrations (27 %) is very similar to that obtained by 10^{-5} M-eserine. Thus 10^{-8}–10^{-6} M-Ro 3–0422 can be used instead of 10^{-5} M-eserine for selective inhibition of the acetocholinesterase of rat-erythrocyte preparations; experiments with 10^{-7} M-Ro 3–0422 and indoxyl propionate or *n*-butyrate as substrate confirm the results given by 10^{-5} M-eserine (Table 3). On increasing the concentration of Ro 3–0422 above 10^{-6} M further inhibition takes place.

Table 4 shows that the acetocholinesterase of rat-erythrocyte preparations hydrolyses corresponding choline and indoxyl esters at very similar if not identical rates.

Table 4. *Rates of hydrolysis by cholinesterase of rat-erythrocyte preparations and plasma*

Figures are calculated from the data in Tables 2 and 3. The rate of hydrolysis of α-naphthyl acetate by organophosphate-sensitive esterase of plasma is too great to allow any conclusion to be drawn about its rate of hydrolysis by plasma cholinesterase. This also applies to the hydrolysis of the indoxyl esters and of α-naphthyl acetate by liver cholinesterase.

CO_2 evolved (μl./10 min./0·1 ml. of erythrocyte preparation or 0·2 ml. of plasma)

	Choline esters		Indoxyl esters		α-Naphthyl esters	
	Erythrocytes	Plasma	Erythrocytes	Plasma	Erythrocytes	Plasma
Acetate	15·6	19·8	15·8	19·2	23·0	—
Propionate	12·8	32·4	12·5	9·8	—	—
Butyrate	0·8	11·3	1·3	0·0	—	—

DFP and E 600. To determine whether the eserine-resistant enzymic hydrolysis of indoxyl esters is accomplished by an organophosphate-sensitive or an organophosphate-resistant esterase, or a mixture of both, the effect of DFP and E 600 on their hydrolysis was investigated. With indoxyl acetate as substrate the effect of increasing concentrations of either of the two organophosphates resembles that of higher concentrations of Ro 3–0422 (Fig. 1), but the inhibition curves for the eserine-resistant hydrolysis show a uniform slope and approx. 20 % of the total hydrolysis is unaffected by mM-DFP or -E 600. On the other hand, the eserine-resistant hydrolysis of indoxyl propionate and *n*-butyrate is inhibited 69 and 98 % respectively by 10^{-5}M-DFP. Since it has been shown that the inhibition of esterases by organophosphates is irreversible under the experimental conditions (Burgen & Hobbiger, 1951; Myers & Mendel, 1953) these results indicate that more than one enzyme is involved in the eserine-resistant hydrolysis of indoxyl esters by rat-erythrocyte preparations, but the participation of an organophosphate-resistant esterase could not be established with certainty.

Hydrolysis by esterases of rat plasma

Table 2 shows that each of the three indoxyl esters is hydrolysed more rapidly than any of the choline esters, but with both types of substrate the rate of hydrolysis decreases in the order propionate, acetate, butyrate.

Eserine. Eserine (10^{-5}M) inhibits the hydrolysis of the choline esters by more than 90 % (Table 3); the same concentration of eserine inhibits the hydrolysis of indoxyl acetate and propionate by 28 and 7 % respectively but has no effect on the hydrolysis of indoxyl butyrate.

Ro 3–0422. This inhibitor is only slightly less potent as an anticholinesterase if rat plasma is used as enzyme source instead of rat erythrocytes. The I_{50} for hydrolysis of acetylcholine by rat plasma is $3{\cdot}5 \times 10^{-10}$M, and 10^{-9}M-Ro 3–0422 reduces cholinesterase activity by 95 % (Fig. 2). With indoxyl acetate as substrate the effect of Ro 3–0422 is similar to that previously found in experiments with rat-erythrocyte preparations. The inhibition curve shows a distinct plateau between 10^{-10} and 10^{-8}M and the inhibition achieved by these concentrations is of the same order as that obtained by 10^{-5}M-eserine (Table 3). Comparable results are also obtained when either 10^{-5}M-eserine or 10^{-8}M-Ro 3–0422 is used in experiments with indoxyl propionate and butyrate as substrate (Table 3).

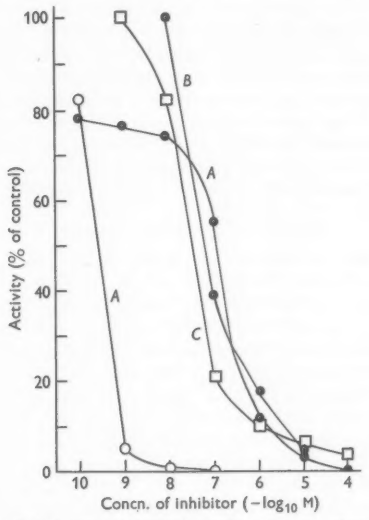

Fig. 2. Inhibition of hydrolysis by plasma after pre-incubation of the enzyme for 20 min. with the inhibitor. Substrate concn. was 6 mM. ○, Acetylcholine; ●, indoxyl acetate; □, α-naphthyl acetate. Curves *A*, Ro 3–0422; *B*, DFP; *C*, DINP.

Hydrolysis of the indoxyl esters, which is inhibited by 10^{-5}M-eserine or 10^{-8}M-Ro 3–0422, can only represent cholinesterase activity. The hydrolysis of the choline esters is known to be accomplished by two different cholinesterases which differ in their substrate specificities; one is an acetocholinesterase and the other a propionocholinesterase (Mundell, 1944; Myers, 1953). B.W. 285C51 (10^{-4}M) was

found to inhibit completely the acetocholinesterase without affecting the propionocholinesterase. However, no attempt has been made to measure the relative rates of hydrolysis of the indoxyl esters by the two cholinesterases since their contribution to the total hydrolysis was too small to allow an accurate assessment of the effect of B.W. 285C51. Plasma cholinesterases hydrolyse indoxyl acetate and propionate but not indoxyl butyrate (Table 4), and thus it seems likely that the acetocholinesterase contributes significantly to their hydrolysis.

The hydrolysis of indoxyl acetate not attributable to cholinesterase(s) can be inhibited almost completely by DFP and concentrations of Ro 3–0422 greater than 10^{-8} M and is therefore due to an organophosphate-sensitive esterase. The inhibition curves obtained with these two organophosphates are continuous but do not entirely exclude the possibility that an organophosphate-resistant enzyme may contribute slightly to the hydrolysis (Fig. 2). With indoxyl propionate and butyrate as substrates 99 % inhibition is obtained by 10^{-5} M-DFP (Table 3).

Hydrolysis by esterase of rat liver

Since de Duve *et al.* (1955) have stated that measurements of enzyme activity of whole homogenates are unreliable, as a result of agglutination which usually occurs when nuclei are present, the nuclear fraction was separated from fraction E (see Methods) and the activity of the whole homogenate determined as the sum of the activities of each of these fractions. The hydrolysis of indoxyl and choline esters is mainly accomplished by fraction E (i.e. the cytoplasm; Table 5) and the activity of the nuclei is largely, if not entirely, due to contamination (Underhay, Holt, Beaufay & de Duve, 1956). This is especially noticeable with the indoxyl esters as substrates, and subsequent experiments in which the enzyme specificity of the esters was investigated were therefore only carried out with fraction E.

Fraction E. Ro 3–0422 in a 10^{-8} M-concentration inhibits the hydrolysis of acetylcholine by 95 %, but has no effect on the hydrolysis of indoxyl acetate (Fig. 3). Thus cholinesterase does not contribute to the hydrolysis of the latter under the experimental conditions used. The enzyme(s) responsible for the hydrolysis of indoxyl acetate is inhibited by E 600, DINP and concentrations of Ro 3–0422 above 10^{-8} M (Fig. 3). The shape of the inhibition curves shows that at least 90 % of the hydrolysis is accomplished by an organophosphate-sensitive enzyme. Since the inhibition curves show a tendency to flatten out at high concentrations of the organophosphate, there is a possibility that a second enzyme (i.e. an organophosphate-resistant esterase) contributes 10 % or less to the total hydrolysis.

With indoxyl propionate or butyrate as substrate the inhibition curves obtained with Ro3–0422 closely resemble those obtained with indoxyl acetate as substrate and these esters are therefore also mainly, if not entirely, hydrolysed by an organophosphate-sensitive esterase.

Eserine in concentrations above 10^{-6} M also inhibits the hydrolysis of indoxyl esters (Fig. 3), although cholinesterase plays no part in it. Thus concentrations of 10^{-4} or 10^{-5} M-eserine, which are widely used for selective inhibition of cholinesterase, do not act as such in this case.

Table 5. *Distribution of activity between the nuclei and cytoplasmic extract of rat-liver homogenates*

Nuclei (N) and cytoplasmic extract (E) of a sucrose-liver homogenate were separated according to the method of de Duve *et al.* (1955). Esterase activities of N and E were determined manometrically at 37° and pH 7·5 and the activity of the whole homogenate was obtained by combining these results in the correct proportion. Standard deviations and the number of determinations are given.

	CO_2 evolved (μl./10 min./100 mg. of liver)			Activity in N (%)
Acetylcholine	16·4	± 2·2	(5)	12·2
Propionylcholine	22·4	± 2·8	(3)	8·7
Butyrylcholine	15·9	± 0·9	(2)	7·9
Indoxyl acetate	4 570	± 440	(5)	5·1
Indoxyl propionate	10 000	± 1 870	(3)	6·4
Indoxyl butyrate	3 900	± 670	(4)	5·6
α-Naphthyl acetate	4 350	± 700	(3)	5·2

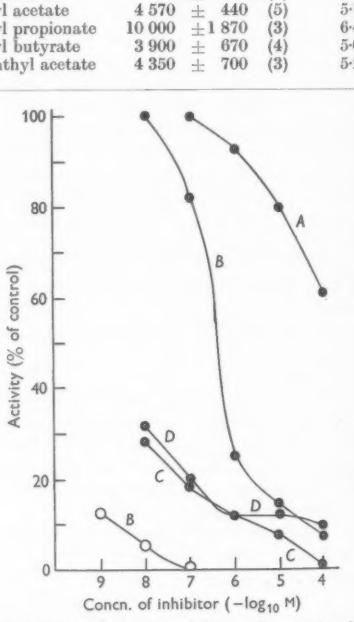

Fig. 3. Inhibition of hydrolysis by liver after pre-incubation of the inhibitor for 20 min. with the enzyme. Substrate concn. was 6 mM. ○, Acetylcholine; ●, indoxyl acetate. Curve *A*, eserine; *B*, Ro 3–0422; *C*, E 600; *D*, DINP.

Hydrolysis of α-naphthyl acetate

The rate of hydrolysis of α-naphthyl acetate is similar to that of indoxyl acetate when either rat-erythrocyte preparations (Table 2) or fraction E or the nuclei of liver homogenates (Table 5) were used as enzyme source. The hydrolysis by rat plasma, however, is approximately five times as great as that of indoxyl acetate (Table 2). α-Naphthyl acetate, like indoxyl acetate, is not a selective substrate for cholinesterases and, with rat-erythrocyte preparations or plasma as enzyme sources, only 40% and less than 10% respectively are attributable to these enzymes (Table 3). DFP (10^{-5} M) inhibits the eserine-resistant hydrolysis by plasma by 99% but has far less effect if erythrocyte preparations are used as enzyme source (Table 3). The effect of organophosphates on the hydrolysis by fraction E is comparable with their effect on the hydrolysis of indoxyl acetate.

DISCUSSION

Previous work with human erythrocytes and human plasma has shown that indoxyl esters are substrates for several types of esterases (Underhay, 1957) and this is confirmed by the present results with rat-enzyme preparations. As the indoxyl esters have very low solubility in 0·025 M-$NaHCO_3$, the experiments had to be carried out in ethanolic solution (final concentration not more than 5%). All the results, and the conclusions drawn from them, apply only to these conditions.

Acetocholinesterase of the rat hydrolyses choline and indoxyl esters at similar if not identical rates (Table 4). Human butyrocholinesterase hydrolyses indoxyl acetate, propionate and butyrate at 30, 29 and 9% respectively of the rate of the corresponding choline esters (Underhay, 1957). No such studies could be carried out with rat propionocholinesterase, which is the equivalent of human butyrocholinesterase (Myers, 1953). The hydrolysis of indoxyl esters by rat plasma, an enzyme preparation which contains propionocholinesterase, is mainly accomplished by esterases other than cholinesterases. Since an acetocholinesterase is also present in rat plasma, selective inhibition of this enzyme would be necessary before the hydrolysis of the indoxyl esters by the propionocholinesterase could be measured. The accuracy required in such experiments cannot be achieved at present.

The major part of the hydrolysis of indoxyl esters by erythrocyte preparations, plasma or liver of the rat must be attributed to esterases which are not cholinesterases (Table 6). The latter have been classified by Aldridge (1953) and by Mounter & Whittaker (1953) according to their sensitivity to organophosphates.

Experiments designed to investigate the effect of different concentrations of organophosphates on the hydrolysis of the indoxyl esters showed that at least 90% of the activity is arrested by 10^{-4} to 10^{-6} M-concentrations of Ro 3-0422, DFP, E 600 or DINP, when either plasma or liver is used as enzyme source, and the major part of the hydrolysis is accomplished by an organophosphate-sensitive esterase(s). The substrate-specificity pattern of this esterase is propionate > acetate ≥ butyrate (Table 6). The hydrolysis of α-naphthyl acetate by plasma or liver is also mainly accomplished by an organophosphate-sensitive esterase but its rate of hydrolysis differs from that of indoxyl acetate. The plasma enzyme hydrolyses α-naphthyl acetate very much faster than indoxyl acetate, whereas for the liver enzyme the rates are similar (Table 6). These results confirm the work of Aldridge (1954), who also found that the plasma and liver enzymes have different substrate specificities, but it was not possible to demonstrate the presence of either more than one organophosphate-sensitive esterase or of

Table 6. *Contributions made by cholinesterase and organophosphate-sensitive esterase towards the total hydrolysis by erythrocyte preparations, plasma and liver homogenates*

Organophosphate-resistant esterase activity could not be established with certainty and is therefore not included. Activity is expressed as μl. of CO_2 evolved/10 min./0·1 ml. of erythrocyte preparation, 0·2 ml. of plasma or 1 mg. of liver homogenate.

	Indoxyl acetate	Indoxyl propionate	Indoxyl butyrate	α-Naphthyl acetate
Erythrocyte preparation				
Cholinesterase	16	12	2	23
Organophosphate-sensitive esterase	45	15	16	34
Plasma				
Cholinesterase	19	10	0	0
Organophosphate-sensitive esterase	53	154	49	366
Liver homogenate				
Cholinesterase	0	0	0	0
Organophosphate-sensitive esterase	46	100	39	44

different esterases in either of these preparations on the basis of inhibition studies or by the use of mixed substrates. Only with indoxyl and α-naphthyl acetates as substrate does an organophosphate-resistant esterase seem to contribute to the hydrolysis, but the contribution by this enzyme cannot be more than 10 % with liver and 5 % with plasma.

The hydrolysis, not attributable to acetocholinesterase, of indoxyl acetate by erythrocyte preparations is about 100 times less sensitive to organophosphates than is that of either plasma or liver. This difference is not the result of enzymic hydrolysis of the organophosphates, as was shown by appropriate control experiments. Since progressive enzyme inhibition was obtained with increasing concentrations of the organophosphates up to mM, it was not possible to establish the extent to which an organophosphate-resistant esterase contributes to the hydrolysis of indoxyl acetate. The inhibition of the hydrolysis of indoxyl propionate and butyrate by organophosphates is even more complete than the inhibition of hydrolysis of indoxyl acetate. Since Myers & Mendel (1953) have shown that the inhibition of esterases by organophosphates is irreversible under the experimental conditions used, this difference in the sensitivity to organophosphates of the organophosphate-sensitive esterase in erythrocyte preparations, when different substrates are used, seems to indicate that more than one enzyme is involved.

All the results as well as their interpretation only apply to the experimental conditions used. For instance, Davies & Rutland (1956) have recently reported that acetylcholine and butyrylcholine are hydrolysed at the same rate by rat-erythrocyte preparations whereas the present investigation shows that butyrylcholine is hydrolysed at only 5 % of the rate of acetylcholine. The reason for this discrepancy is that Davies & Rutland used higher substrate concentrations (0·015M for acetylcholine and 0·03M for butyrylcholine; Dr D. R. Davies, personal communication) and with these concentrations identical results were obtained in this Laboratory. In the present work it was also found that the presence of 5 % of ethanol inhibits the hydrolysis of 0·03M-butyrylcholine by rat-erythrocyte preparations by 80 %, although the same concentration of ethanol has no effect on the hydrolysis of acetylcholine by erythrocyte preparations or, incidentally, of butyrylcholine by plasma.

During this work Ro 3–0422 has been successfully used as a selective inhibitor for cholinesterases. Hobbiger (1954) reported that this compound is more potent than any other organophosphate in inhibiting human cholinesterases and, furthermore, that its effect, like that of other organophosphates with a diethyl- or di*iso*propyl-phosphoryl group, is not reversed by substrate. Hobbiger, Simpson & Tait (1954) found that Ro 3–0422 is a more selective anticholinesterase than tetraethyl pyrophosphate (TEPP) or DFP. It has now been found that this also applies to rat cholinesterases and that these enzymes have a 100–1000-fold greater sensitivity to Ro 3–0422 than other esterases in the rat. Ro 3–0422, unlike other organophosphate inhibitors, can thus be used instead of eserine as a selective inhibitor of cholinesterases over a wide range of concentrations. The experiments with rat liver show that this substance is an even more selective anticholinesterase than eserine. Ro 3–0422 inhibits the cholinesterases of rat liver in concentrations which have no effect on other esterases, whereas eserine does not act as a selective inhibitor in this case although it can be used as such with rat-erythrocyte preparations or plasma. Similarly, Myers (1953) reported that the hydrolysis of butyrylcholine by Harderian gland of the rat could be inhibited by 10^{-6}–10^{-4}M-concentrations of eserine although another potent anticholinesterase Nu 683 [dimethyl carbamate of (2-hydroxy-5-phenyl)benzyltrimethylammonium bromide] had no effect on it.

SUMMARY

1. Unsubstituted indoxyl esters are hydrolysed by rat-erythrocyte preparations, plasma and liver homogenates at a considerably greater rate than any of the corresponding choline esters.

2. The enzymes chiefly responsible for the hydrolysis of the indoxyl esters are organophosphate-sensitive esterases. Under the experimental conditions described, cholinesterases of erythrocyte preparations and plasma contributed only a fraction of the total hydrolysis, and any contribution by liver cholinesterase was too small to be detected. An organophosphate-resistant esterase may contribute to an extent of not more than 10 % in each case.

3. The organophosphate Ro 3–0422 is a more selective anticholinesterase than eserine and has been successfully used as such with all three enzyme preparations.

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The Metabolism of 2:3-, 2:6- and 3:5-Dichloronitrobenzene and the Formation of a Mercapturic Acid from 2:3:4:5-Tetrachloronitrobenzene in the Rabbit

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Previous papers (Bray, Hybs, James & Thorpe, 1953; Betts, James & Thorpe, 1955; Bray, James & Thorpe, 1956, 1957; Betts, Bray, James & Thorpe, 1957) have described the metabolism in the rabbit of all members of the chloromononitrobenzene series except 2:3-, 2:6- and 3:5-dichloronitrobenzene. One aspect of the metabolism of these compounds of particular interest is the readiness with which mercapturic acids are formed by most members of the series. Furthermore, these mercapturic acids are all formed, in effect, either by acetylcysteyldenitration or by acetylcysteyldechlorination, and not by acetylcysteyldeprotonation as had been observed in all previous instances of the formation of nuclear mercapturic acids (for references see Bray *et al.* 1956). The present paper describes the metabolic fate of 2:3-, 2:6- and 3:5-dichloronitrobenzene. No conclusive evidence has been obtained for the formation of mercapturic acid by these three compounds.

The early work on tetrachloronitrobenzenes (Bray *et al.* 1953) was published before an example of nuclear acetylcysteyldechlorination had been discovered. When this type of mercapturic acid formation was observed (Bray *et al.* 1956, 1957; Betts *et al.* 1957) it became necessary to re-examine the tetrachloronitrobenzenes to see whether a product of acetylcysteyldechlorination had been overlooked, especially with 2:3:4:5-tetrachloronitrobenzene, from which no mercapturic acid had been obtained. The search for a mercapturic acid formed by elimination of chlorine was facilitated by the fact that the resulting mercapturic acid retained the nitro group and, therefore, could be more readily detected by its colour in alkaline solution if only small amounts were present. It has, in fact, now been shown that 2:3:4:5-tetrachloronitrobenzene does form a small amount of mercapturic acid by acetylcysteyldechlorination. The previous finding that no *N*-acetyl-*S*-(2:3:4:5-tetrachlorophenyl)-L-cysteine is formed has been confirmed.

MATERIALS

All melting points recorded are uncorrected. Elementary microanalyses were carried out by F. and E. Pascher, Bonn.

2:3- and 3:5-Dichloronitrobenzene were the gift of Bayer Products Ltd. Some 3:5-dichloronitrobenzene as well as 2:6-dichloronitrobenzene was obtained by the method of Holleman (1904).

2:3-Dichloroaniline was purchased; 2:6- and 3:5-dichloroaniline, m.p. 49° and 39° respectively, were prepared by reduction of the nitro compounds. The three bases were converted into the corresponding dichloroacetanilides, m.p. 159°, 174° and 187° respectively. The *picrate* of 2:6-dichloroaniline formed orange needles from water, m.p. 89° (Found: 2:6-dichloroaniline, 42·5. $C_{12}H_9O_7N_4Cl_2$ requires 2:6-dichloroaniline, 41·2%).

3:5:3′:5′-*Tetrachloroazoxybenzene* was prepared by boiling 3:5-dichlorophenylhydroxylamine in 96% (w/v) ethanol for 15 min. according to the method of Haworth & Lapworth (1921). It formed fine colourless threads, m.p. 168°, from aqueous ethanol (Found: C, 42·7; H, 1·7; N, 8·3; Cl, 42·7. $C_{12}H_6ON_2Cl_4$ requires C, 42·9; H, 1·8; N, 8·3; Cl, 42·2%).

N-*Acetyl*-S-(2-*chloro*-6-*nitrophenyl*)-L-*cysteine*. This was prepared from 2-chloro-6-nitroaniline by the method of Parke & Williams (1951) as modified by Bray *et al.* (1956). 2-Chloro-6-nitroaniline was obtained by hydrolysis of 2-chloro-6-nitroacetanilide prepared as described by Franzen & Engel (1921). The mercapturic acid was also made by acetylation (cf. Zbarsky & Young, 1943) of *S*-(2-chloro-6-nitrophenyl)-L-cysteine obtained by interaction of 2:3-dichloronitrobenzene and cysteine in alkaline solution (Suter, 1895). N-*Acetyl*-S-(2-*chloro*-6-*nitrophenyl*)-L-*cysteine* formed pale-yellow rectangular plates from water, m.p. 185°

(decomp.), $[\alpha]_D^{23}$ in ethanol $+72 \pm 5°$ (c, 0·31); light-absorption max. in 0·1 N-KOH, 260 mμ (ϵ, 4776), min., 241 mμ (Found: C, 41·6; H, 3·7; N, 8·9; Cl, 11·3; S, 9·7%; equiv. 310. $C_{11}H_{11}O_5N_2ClS$ requires C, 41·5; H, 3·5; N, 8·8; Cl, 11·1; S, 10·1%; equiv. 319).

3:4-Dichloro-2-nitrophenol, m.p. 76°, was prepared by the method of Hodgson & Kershaw (1929), 3:5-dichloro-4-nitrophenol, m.p. 146–148°, by that of Hodgson & Wignall (1927), 2:4-dichloro-3-nitrophenol, m.p. 70°, by that of Groves, Turner & Sharp (1929) and 2:4-dichloro-6-nitrophenol, m.p. 121–122°, by the action of $KClO_3$ and HCl on *o*-nitrophenol (cf. Holleman, 1904). 2:6-Dichloro-4-nitrophenol, m.p. 123°, was purchased. The melting points of these compounds were substantially in agreement with those given in the literature.

Aminodichlorophenols were prepared by the action of alkaline dithionite on the corresponding dichloronitrophenols except 3:4-dichloro-2-nitrophenol, which did not yield the expected 2-amino-3:4-dichlorophenol. *3-Amino-2:4-dichlorophenol* formed colourless needles from water, m.p. 83° (Found: N, 7·8; Cl, 39·7. $C_6H_5ONCl_2$ requires N, 7·9; Cl, 40·1%). This compound reacted abnormally with the Bratton & Marshall (1939) reagents (see Results section). At the nitrous acid stage it gave a very intense yellow colour, although the addition of the coupling reagent produced an immediate magenta colour. The yellow colour could be readily distinguished from that of the diazo oxides formed by *o*-aminophenols not only by the greater intensity of the colour but also by the rapidity with which the red colour is formed when the coupling reagent is added. With *o*-aminophenols the formation of the azo dye is relatively slow and the colour intensity is relatively weak. The melting points of 4-amino-3:5-dichlorophenol and 4-amino-2:6-dichlorophenol agreed with those previously recorded, but 2-amino-4:6-dichlorophenol (Found: N, 8·4. $C_6H_5ONCl_2$ requires N, 7·9%) had m.p. 92°. Hunter & Barnes (1928) give 109° (decomp.).

METHODS

Animals, diet and dosage. Doe rabbits (2–3 kg. body wt.) were maintained as described by Bray, Ryman & Thorpe (1947). Compounds were administered by stomach tube as suspensions in water. The dose levels were: 2:3-isomer, 0·1 g./kg.; 2:6-isomer, 0·3 g./kg.; 3:5-isomer, 0·2 g./kg. Dose levels above these were usually fatal; even the stated dose level for the 3:5-isomer was occasionally fatal.

Determination of metabolites

The methods used for determination of ethereal sulphate, glucuronic acid, reducing material, mercapturic acid, dichloroanilines and catechols were as given by Bray *et al.* 1957. The concentration of NaOH used for hydrolysis of mercapturic acid was 2 N and the time of boiling was 1 hr. Recoveries of dichloroanilines added to urine were 97, 69, and 100% from the 2:3-, 2:6- and 3:5-isomers respectively.

Qualitative examination

Faeces. Steam-distillates of the 72 hr. faeces were examined for unabsorbed dichloronitrobenzene and dichloroaniline by determination of dichloroanilines before and after reduction.

Urine. Ether extracts A, B, C and D were prepared from urines as described by Bray *et al.* (1956). Briefly these were: A, from urine at pH 7–8; B, from the residual urine from A adjusted to pH 1; C, from the residual urine from B hydrolysed with an equal volume of 10 N-H_2SO_4; D, from residual urine from C adjusted to pH 7.

Paper chromatography. The procedure was as described by Bray, Thorpe & White (1950) except that to minimize decomposition of aminophenols papers were dried without heating. The solvents and detecting reagents used and the R_F values of reference compounds are given in Table 3. For the application of the indophenol test (Thorpe, Williams & Shelswell, 1941) papers were sprayed with 0·5 N-Na_2CO_3, to which bromine had been added until just yellow, followed by 5% (w/v) aqueous phenol. The sodium hypobromite was freshly prepared and used within 1 hr. *p*-Aminophenols give a deep-blue colour changed to red by spraying with N-acetic acid.

In addition to the direct examination for mercapturic acids on paper chromatograms, extracts B of the urines were hydrolysed by refluxing with N-NaOH for 1 hr. and steam-distilled. The distillates were examined for thiophenols by paper chromatography as described by Parke & Williams (1955).

Isolation of metabolites

Unless stated otherwise all metabolites isolated were shown to be identical with synthetic specimens, by mixed m.p. and, except 3:5:3′:5′-tetrachloroazoxybenzene, by paper chromatography. The methods were essentially as described by Bray *et al.* (1957).

Mercapturic acid from 2:3:4:5-tetrachloronitrobenzene

The 24 hr. urine of five rabbits, which had each received 0·7 g. of 2:3:4:5-tetrachloronitrobenzene, was acidified (pH 1) and continuously extracted with ether. The acids were separated from the ethereal solution by extraction with 0·5 N-$NaHCO_3$. The aqueous extract was acidified and extracted with ether. The syrup obtained on evaporation of the solvent from the ether extract was dissolved in ethanol, placed on a column of Whatman paper powder (34 cm. long, 3 cm. diameter) and eluted with the upper layer of solvent mixture *C* (Table 3). That fraction of the eluate which was yellow was washed with water and evaporated to dryness under reduced pressure. The residue was dissolved in acetone and water was added until a turbidity developed. A small amount of charcoal was added and the solution was filtered. The crystals which separated from the filtrate were recrystallized from aqueous acetone and gave 27 mg. (0·5% of dose) of sheaves of yellow needles, m.p. 178° (decomp.), light-absorption max. 267 mμ (ϵ, 6103), min., 257 mμ (Found: N, 7·2; Cl, 26·7. $C_{11}H_9O_5N_2Cl_3S$ requires N, 7·2; Cl, 27·5%).

RESULTS

The average daily excretions of normal metabolites by the rabbits used were of the same order as those found previously (e.g. Bray *et al.* 1953). The results obtained from quantitative analysis of the urines of rabbits dosed with dichloronitrobenzenes are summarized in Table 1 which also shows that about 5% of a dose of 2:3- and 3:5-dichloronitrobenzene and 1% of a dose of 2:6-dichloronitrobenzene is unabsorbed, the greater part of the unabsorbed material being reduced to the aniline. None of the

Table 1. *Excretion of metabolites of* 2:3-, 2:6- *and* 3:5-*dichloronitrobenzene by the rabbit*

Results are expressed as percentages of the dose, given as means with ranges in parentheses; superior figures indicate the number of experiments. Urine was collected for 72 hr. except in experiments (indicated by superior m) where separate consecutive samples of urine, which were collected from rabbits given water at intervals (see Bray, Thorpe & White, 1951), were analysed until excretion of metabolites could no longer be detected. — Indicates not determined.

	2:3-Dichloronitrobenzene		2:6-Dichloro-nitrobenzene	3:5-Dichloronitrobenzene	
Dose (g.)	0·3	0·3	1·0	0·6	0·5
Metabolite					
Unabsorbed					
Dichloronitrobenzene	—	1·5 (0, 3)[2]	0·5	—	0·4 (0·2, 0·6)[2]
Dichloroaniline	—	3·5 (2, 5)[2]	0·35 (0·4, 0·3)[2]	—	4·0 (3, 5)[2]
Mercapturic acid	13[m]* (12–14)[3]	—	0 (0)[5]	0 (0)[3]	0 (0)[3]
Glucosiduronic acid	33[m] (30, 36)[2]	45 (11–90)[5]	45 (27–64)[4]	59 (29–106)[3]	76 (62–80)[3]
Copper-reducing material (as glucuronic acid)	—	24 (12–35)[5]	22 (11–27)[4]	—	48 (44–56)[3]
Ethereal sulphate	16[m] (6–24)[3]	—	10 (7–15)[3]	8 (4–15)[5]	—
Dichloroaniline, free	—	14 (7–17)[9]	4 (2–7)[5]	32 (22–45)[15]	24 (16–32)[3]
Dichloroaniline, combined	—	5 (3–8)[3]	0·1†	0	—
'Catechols'	—	10‡ (7–15)[3]	—	—	—
Azoxy compound	—	0	0	>2	>2

* It is very doubtful whether this represents mercapturic acid (see text).
† On pooled urine from two rabbits. ‡ For interpretation see text.

three dichloronitrobenzenes was found in urine in the form of mercapturic acid. The urine from animals given 2:3-dichloronitrobenzene had an uptake of iodine by the Stekol (1936) method equivalent to a conversion of about 13 % of the dose into mercapturic acid, but no evidence for the presence of a mercapturic acid could be obtained even by paper chromatography (see below). If such an amount had been present it should have been possible to isolate some of it, especially as a synthetic specimen of the expected mercapturic acid was available as a guide to its properties. The explanation of the iodine uptake is unknown. With 3:5-dichloronitrobenzene the urine had a marked uptake of iodine before, but not after, alkaline hydrolysis. The excretion of a large proportion of the dose as glucosiduronic acid and ethereal sulphate supports the expectation that some of each isomer would be hydroxylated. Approximately half of the glucuronic acid was excreted in a form which reduced alkaline cupric solutions, but there is no evidence to show whether this is due to an *N*-glucosiduronic acid (cf. Boyland, Manson & Orr, 1957) or a labile ether-type glucosiduronic acid. With the 2:6-isomer large quantities of an *N*-glucosiduronic acid are ruled out because only 4 % of the dose was excreted as dichloroaniline. The ethereal sulphate is presumably formed from amino- or nitro-dichlorophenols. With the 2:3- and 3:5-isomers considerable reduction occurred and most of the base was excreted as such. 2:3-Dichloronitrobenzene was the only isomer from which significant amounts of the aniline were excreted combined. The 'catechol' determination, with 4-chlorocatechol as standard, gave results corresponding to an excretion of 10 % of the dose of 2:3-dichloronitrobenzene as 'catechol'. No catechol derivative, however, could be detected in the urine by paper chromatography. The probable explanation is that the blue colour obtained with the reagents of Azouz, Parke & Williams (1953) is caused by 4-amino-2:3-dichlorophenol, since an extract D, in which this aminophenol but no catechol was detected, gave a blue colour when treated with alkali (see Table 3) or with the reagents of Azouz *et al.* (1953) under the conditions for determination of catechol. With 2:6-dichloronitrobenzene, from which a catechol derivative was identified as a metabolite, the quantitative method could not be applied since 4-amino-3:5-dichlorophenol gave a calibration curve

with a slope half of that obtained with 4-chlorocatechol. 4-Amino-2:3:5-trichlorophenol also gives a blue colour with the reagents so that results obtained by the method of Azouz *et al.* (1953) should be accepted with caution when aminophenols may be present.

Metabolites isolated or detected

The metabolites isolated and the yields obtained are listed in Table 2 and the metabolites detected by paper chromatography in extracts A, B, C and D of the urines are given in Table 3. Authentic specimens

Table 2. *Metabolites isolated from urine of rabbits given* 2:3-, 2:6- *and* 3:5-*dichloronitrobenzene*

Solvents for recrystallization: E, ethanol; W, water. Those given in parentheses are for the derivative isolated.

Nitrobenzene derivative administered	Dose/rabbit (g.)	No. of rabbits	Compound isolated	Solvent	Yield (% of dose)
2:3-Dichloro-	0·3	6	2:3-Dichloroaniline (as acetyl deriv.)	(WE)	5
2:6-Dichloro-	1·0	3	2:6-Dichloroaniline (as picrate)	(W)	2
	1·0	3	4-Amino-3:5-dichlorophenol	W	7
3:5-Dichloro-	0·6	3	3:5-Dichloroaniline (as acetyl deriv.)	(WE)	3
	0·5	6	3:5:3′:5′-Tetrachloroazoxybenzene	E	2
	0·6	6	2-Amino-4:6-dichlorophenol	W	0·6
	0·8	10	4-Amino-2:6-dichlorophenol	WE	0·1

Table 3. R_F *values and detection of some possible metabolites of* 2:3-, 2:6- *and* 3:5-*dichloronitrobenzene*

Solvent mixtures and times of run on Whatman no. 4 paper: *A*, benzene–acetic acid–water (2:2:1, by vol.) (1·5 hr.); *B*, light petroleum (b.p. 90°)–benzene–98% formic acid (3:1:2, by vol.) (1 hr.); *C*, butanol–3N-$(NH_4)_2CO_3$–aq. 3N-NH_3 soln. (4:3:3, by vol.; Corner & Young, 1954) (2 hr.). Detecting reagents: *a*, 2N-HCl, 0·1% $NaNO_2$, 0·5% ammonium sulphamate and 0·1% *N*-(1-naphthyl)ethylenediamine dihydrochloride (Bratton & Marshall, 1939); *b*, diazotized *p*-nitroaniline followed by 20% (w/v) Na_2CO_3 (Bray *et al.* 1950); *c*, 20% (w/v) Na_2CO_3; *d*, indophenol reaction (see Methods). Colours of spots: B, blue; Bn, brown; G, green; Gy, grey; M, magenta; O, orange; P, purple; R, red; Y, yellow; d, deep; p, pale; vp, very pale. Synthetic specimens of the compounds in parentheses were not available for comparison; the arguments for the suggested constitutions are given in the Results section.

Metabolites were sought in extracts A, B, C and D (see Methods). Compounds found in extracts of hydrolysed urine (C and D) are probably excreted in combined form. +, Present; (+), present in traces; −, absent.

	R_F values in solvent mixture			Colour with detecting reagent				Detected in extract			
	A	*B*	*C*	*a*	*b*	*c*	*d*	A	B	C	D
Derivatives of 2:3-dichloronitrobenzene											
(2-Amino-3:4-dichlorophenol)	0·90	0·15*	0·90	vpR†	—	—	—	−	(+)	(+)	(+)
(3-Amino-4:5-dichlorophenol)	0·71	0·25*	0·82	M	—	—	—	(+)	(+)	+	−
(4-Amino-2:3-dichlorophenol)	0·77	0·14*	0·72	B	pG	B	B	(+)	−	(+)	+
3:4-Dichloro-2-nitrophenol	1·0	1·0	0·83	—	—	Y	—	−	−	−	−
(4:5-Dichloro-3-nitrophenol)	1·0	0·50	0·68	—	—	Y	—	+	(+)	(+)	−
N-Acetyl-*S*-(2-chloro-6-nitrophenyl)-L-cysteine	—	0·0	0·46	—	—	vpY‡	—	−	−	−	−
2:3-Dichloroaniline	1·0	1·0	0·94	M	pY	—	—	+	+	(+)	−
Derivatives of 2:6-dichloronitrobenzene											
3-Amino-2:4-dichlorophenol	1·0	0·80*	0·72	M§	RBn	—	GyBn	−	−	−	−
4-Amino-3:5-dichlorophenol	0·88	0·15*	0·90	M	pG	GyB	B	+	+	+	+
2:4-Dichloro-3-nitrophenol	—	0·78*	0·81	—	—	Y	—	+	−	−	−
3:5-Dichloro-4-nitrophenol	—	0·43*	0·75	—	—	Y	—	−	−	−	−
(3:5-Dichloro-4-nitrocatechol)‖	0·52	0·0	0·53	—	—	pO	—	−	(+)	−	−
2:6-Dichloroaniline	1·0	1·0	0·90	pOR	—	—	—	+	−	−	−
Derivatives of 3:5-dichloronitrobenzene											
2-Amino-4:6-dichlorophenol	1·0	0·0	0·72	vpR†	BnG	Bn	Bn	−	+	+	+
4-Amino-2:6-dichlorophenol	0·55	0·15	0·50	B	pG	Gy	B	(+)	−	(+)	+
2:4-Dichloro-6-nitrophenol	1·0	1·0	0·72	—	—	dY	—	−	−	−	−
2:6-Dichloro-4-nitrophenol	0·96	0·60	0·59	—	—	Y	—	−	−	−	−
3:5-Dichloroaniline	1·0	1·0	0·86	M	—	—	—	+	+	(+)	−

* Approximate value; elongated spots.
† Bright yellow with nitrous acid.
‡ Black in ultraviolet light.
§ Intense yellow with nitrous acid (see Materials section).
‖ Detected by 0·2% $FeCl_3$, which gave typical catechol green turned red by NH_3.

of the possible aminophenols from 2:3-dichloronitrobenzene were not available but the structure of the aminophenols can be attributed with fair certainty on the basis of the chemical tests which distinguish between *o*-, *m*- and *p*-aminophenols. *o*-Aminophenols give a bright-yellow diazo oxide colour at the nitrous acid stage of the Bratton & Marshall (1939) test and the final colour appears slowly, *m*-aminophenols give usually a magenta colour immediately with the Bratton & Marshall reagents and *p*-aminophenols give usually a bluish colour slowly. *p*-Aminophenols are readily identified by the indophenol test (see Methods). Only the following metabolites require comment.

From 2:3-*dichloronitrobenzene*. There are three possible aminodichlorophenols and an *o*-, an *m*- and a *p*-aminophenol were detected. It is probable that these aminophenols have the constitutions shown in Table 3 since the only other phenols which could be confused with these would be those formed by hydroxyldechlorination (cf. Betts *et al.* 1957) of 2:3-dichloroaniline, i.e. either 2-amino-6-chlorophenol or 3-amino-2-chlorophenol. R_F values (Bray *et al.* 1956) distinguish the former (0·8 in solvent *A*) from 2-amino-3:4-dichlorophenol (0·9). 3-Amino-2-chlorophenol has almost the same R_F value (0·7) in solvent *A* as has 3-amino-4:5-dichlorophenol (0·71) but in chloroform–acetic acid–water (2:1:1, by vol.; 2·2 hr.) the R_F values are 0·91 and 1·0 respectively. Furthermore, it is unlikely that 3-amino-2-chlorophenol would be formed since this would involve the replacement of chlorine in the *meta* position which is well known to be stable.

The structure attributed to 4:5-dichloro-3-nitrophenol is based upon the formation of a phenol identical with the 3-amino-4:5-dichlorophenol when spots of the nitrophenol were eluted from the chromatograms with aqueous ethanol and reduced with tin and hydrochloric acid. This nitrophenol (R_F 0·50 and 0·68 in solvents *B* and *C* respectively) was not 2-chloro-3-nitrophenol, which has R_F values of 0·75 and 0·69 in these solvents (Bray *et al.* 1956).

The abnormal iodine uptake of the hydrolysed urine of animals given 2:3-dichloronitrobenzene (see above) prompted a particularly careful search for mercapturic acid. *N*-Acetyl-*S*-(2-chloro-6-nitrophenyl)-L-cysteine was not detected on any paper chromatograms. A chromatogram was run from extract B with solvent *C*; strips were cut and eluted with ethanol but no sulphur was detected in any of the eluates. It was concluded, therefore, that the increased iodine uptake was not caused by mercapturic acid.

From 2:6-*dichloronitrobenzene*. No mercapturic acid was detected by the methods used above. Synthetic specimens were available for comparison with all the metabolites listed except the catechol. The products of hydroxyldechlorination would be 3-chloro-2-nitrophenol and 2-amino-3-chlorophenol. The R_F values of the former (1·0 and 0·69 in solvents *B* and *C* respectively, Bray *et al.* 1956) are different from those of 2:4-dichloro-3-nitrophenol (0·81 and 0·75) and 2-amino-3-chlorophenol is an *o*-aminophenol whereas only a *p*-aminophenol was detected. Since the catechol gave an orange colour with alkali and no colour with the Bratton & Marshall reagents it was probably a nitrocatechol. The only nitrocatechol which could be formed from 2:6-dichloronitrobenzene is 3:5-dichloro-4-nitrocatechol.

Paper chromatograms of extracts B detected with the Bratton & Marshall reagents showed a magenta spot, R_F 0·22 in solvent *C*. (There was no movement with solvents *A* or *B*.) The aminophenols which could be formed from 2:6-dichloronitrobenzene have R_F values much greater than 0·22. By elution with water of strips cut from a paper chromatogram it was shown that the zone around R_F 0·22 contained material which gave a naphthoresorcinol reaction corresponding in intensity with that of the colour obtained with the Bratton & Marshall test. After an eluate had been hydrolysed with 5N-H_2SO_4 at 100° for 1 hr. examination by paper chromatography revealed no spot at R_F 0·22 but one at R_F 0·90 which reacted in the same way as 4-amino-3:5-dichlorophenol. It was concluded that the material causing the original spot at R_F 0·22 was probably 4-amino-3:5-dichlorophenyl glucosiduronic acid and that this acid was sufficiently soluble in ether to be extracted in extracts B.

From 3:5-*dichloronitrobenzene*. No mercapturic acid was detected and all the metabolites found were identified by comparison with synthetic specimens. The 2-amino-4:6-dichlorophenol had m.p. 92° (see Materials). The products of hydroxyldechlorination would be 3-chloro-5-nitrophenol and 3-amino-5-chlorophenol. No nitrophenol was detected and 3-amino-5-chlorophenol is an *m*-aminophenol whereas only an *o*- and a *p*-aminophenol were detected.

From 2:3:4:5-*tetrachloronitrobenzene*. The properties and elementary analysis of the mercapturic acid isolated were in accord with those of an *N*-acetyl-*S*-(trichloronitrophenyl)-L-cysteine formed by acetylcysteyldechlorination. Since chlorine in a *meta* position to the nitro group is the most stable (see Bunnett & Zahler, 1951) and no labile *meta* chlorine atom has been found in any member of the chloromononitrobenzene series (see Betts *et al.* 1957), it is reasonable to conclude that the acetylcysteyl group would replace the chlorine in either position 2 or position 4. Two observations make the position 4 unlikely. The mercapturic acid does not give, when heated with alkali, the intense yellow colour which is typical of all known mercapturic acids with a nitro group *para* to the acetylcysteyl group (see Betts *et al.* 1957). These mercapturic acids

also all have a light-absorption maximum in the region of 340 mμ (see Betts *et al.* 1957). The absorption spectrum of the mercapturic acid isolated from 2:3:4:5-tetrachloronitrobenzene has no maximum in this region and resembles the spectra of mercapturic acids with a nitro group in the *ortho* position. It is, therefore, reasonable to conclude that the acid isolated was *N*-acetyl-*S*-(2:3:4-trichloro-6-nitrophenyl)-L-cysteine.

From penta- and 2:3:4:6- *and* 2:3:5:6-*tetra-chloronitrobenzene* (cf. Bray *et al.* 1953; Betts *et al.* 1955). A re-examination by paper chromatography of urines from rabbits given these compounds failed to reveal the presence of mercapturic acids containing a nitro group.

DISCUSSION

2:3-, 2:6- *and* 3:5-*Dichloronitrobenzene*

The most conspicuous difference between the metabolic fates of the three dichloronitrobenzenes now reported and those of the three previously examined (Bray *et al.* 1957) is that none of the former appears to yield mercapturic acid. This may be correlated with the stability of the chlorine atoms in these isomers. In the 3:5-isomers the two *meta* chlorine atoms are stable and the values for the mobilities of the labile chlorine atoms in the 2:3- and 2:6-isomers are lower than those for the other three dichloronitrobenzenes (see Table 4). Presumably the nitro groups are as stable as the chlorine atoms. In the absence of mercapturic acid formation, the main metabolic pathways of 2:3-, 2:6- and 3:5-dinitrochloronitrobenzene are reduction and hydroxylation. Less than 5 % of a dose remains unabsorbed.

Reduction. All the isomers are reduced to the corresponding dichloroanilines. The extent of this reduction with the 2:6-isomer (4 % of dose) is less than for any member of the chloromononitrobenzene series. Only 2:3-dichloroaniline was excreted in significant amounts in combined form. 3:5-Dichloronitrobenzene provides another example of a nitro compound which is partly excreted as the corresponding azoxy compound. The origin of the corresponding compound from 3:4-dichloronitrobenzene has been discussed by Bray *et al.* (1957).

Hydroxylation. The hydroxylation products detected are summarized in Fig. 1. The metabolic pathways shown are based on the assumption that hydroxylation of a dichloronitrobenzene will occur in a *meta* position to the nitro group and hydroxylation of a dichloroaniline will occur either *ortho* or *para* to the amino group. This assumption has justification on general chemical grounds and from previous findings in the chloronitrobenzene series (e.g. Bray *et al.* 1957; Betts *et al.* 1957) as well as from the compounds identified in urine. It has been shown (Betts *et al.* 1957) that 2:4:5- and 2:4:6-trichloronitrobenzene both yield aminophenols which have been formed by substitution of one chlorine atom by a hydroxyl group in addition to aminophenols formed by the usual (direct) hydroxylation of an unsubstituted position. No dichloronitrobenzene, however, forms an aminophenol by hydroxyldechlorination. 2:3-Dichloronitrobenzene forms the two aminophenols which would be expected by hydroxylation of 2:3-dichloroaniline as well as that which would be expected by reduction of the nitrophenol formed by direct hydroxylation of the dichloronitrobenzene. This nitrophenol was detected in the urine.

With 2:6-dichloronitrobenzene two aminophenols would be expected, one from the aniline and one from the *m*-nitrophenol. The former was detected but not the latter, although the corresponding nitrophenol was shown to be present in small amount. 2:6-Dichloronitrobenzene is the only member of the series which yielded evidence for the excretion of a catechol derivative. With 3:5-dichloronitrobenzene no *m*-nitrophenol is possible and the two aminophenols expected from 3:5-dichloroaniline were identified. Thus, with the exception of 3-amino-2:4-dichlorophenol, all the aminodichlorophenols expected from the three dichloronitrobenzenes have been identified in urine.

The chloromononitrobenzene series

The main features of the metabolism of the 19 members of the chloromononitrobenzene series have been summarized in Table 4. The most interesting is that 15 members form mercapturic acids in a way not previously observed, that is, in effect, either by acetylcysteyldenitration or nuclear acetylcysteyldechlorination. [Benzyl chloride (Stekol, 1939) undergoes acetylcysteyldechlorination in the side chain.] There is a fairly close correlation between the type of acid formed and the lability of the displaced chloro or nitro groups to nucleophilic substitution reagents as measured by the extent of the hydrolysis of the groups by sodium methoxide or ethanolic sodium hydroxide. Qualitatively the only exception appears to be 2:4:5-trichloronitrobenzene, in which the chlorine in position 2 is replaced, whereas sodium methoxide replaces the chlorine in position 4. A chlorine atom in an *ortho* position in halogenonitrobenzenes is, however, usually more readily displaced by amine reagents than is a chlorine atom in the *para* position (Bunnett & Zahler, 1951), and Beilstein & Kurbatow (1879) found that ethanolic ammonia at 200° displaced the chlorine in position 2 from 2:4:5-trichloronitrobenzene. In the rabbit, chlorine is not displaced from the *para* position of any chloronitrobenzene in which there is an *ortho* chlorine atom (see Table 4). The displacement of chlorine in the rabbit may, therefore, resemble that by amine reagents more

closely than that by sodium methoxide. Quantitative data obtained with the former reagents are not, however, available. The positions of the chlorine atoms displaced by sodium methoxide from tetra- and penta-chloronitrobenzenes are not known.

All the ten mercapturic acids formed by acetylcysteyldechlorination are excreted as nitro compounds. No *N*-acetyl-*S*-(aminochlorophenyl)-L-cysteine was ever detected. Several chloroanilines were fed to rabbits but none caused the formation of mercapturic acids.

All the five chloronitrobenzenes which have chlorine atoms in position 2, 6 and at least one other position form mercapturic acids by acetylcysteyldenitration. That chlorine substituted into the *meta* position has a greater influence on the mobility of the nitro group than has chlorine in a *para* position (Bunnett & Zahler, 1951) is well illustrated by the extent of the formation of mercapturic acid by these five compounds. The four which have chlorine in the *meta* position readily lose the nitro group and yield mercapturic acids (10–37 % of dose), whereas 2:4:6-trichloronitrobenzene, which has chlorine in the *para* but not in the *meta* position, is more resistant to ethanolic sodium hydroxide and forms only a very small amount of mercapturic acid (Table 4). 2:6-Dichloronitrobenzene, which has no chlorine in either *meta* or *para* position, does not yield a mercapturic acid.

The anilines formed by reduction of the chloronitrobenzenes are of interest in that, although many appeared to be excreted partly in conjugated form, in no instance could proof be obtained that acetyl derivatives were excreted. The anilines were excreted mainly in uncombined form and 2:4:6-trichloro-, 2:3:4:6-tetrachloro- and pentachloronitrobenzene were the only compounds from which

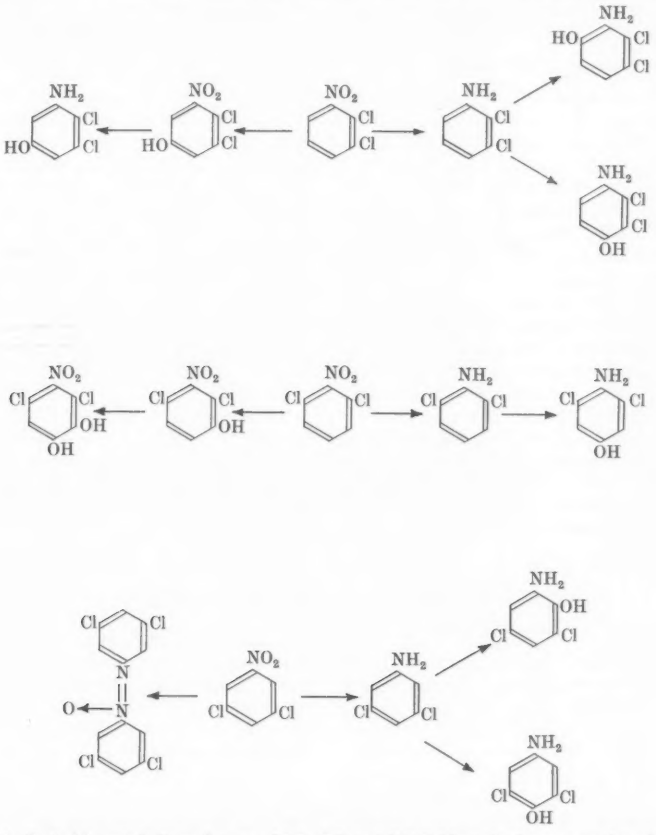

Fig. 1. Probable metabolic pathways of 2:3-, 2:6- and 3:5-dichloronitrobenzene in the rabbit.

Table 4. *Summary of the metabolism of the* 19 *chloromononitrobenzenes*

The values for the average amounts of metabolites excreted are expressed as percentage of the absorbed dose (for details of methods, dosage, and ranges see papers cited). The positions of labile chlorine atoms (indicated in bold type) and their mobilities, m_{Cl} (rate coefficient for hydrolysis with sodium methoxide at 25°), are based on those given by Holleman & van Haeften (1921) and Holleman, de Mooy & Weel (1915). The lability of nitro groups, m_{NO_2}, is based on liberation of NO_2^- ions with ethanolic NaOH at 100° (Betts *et al.* 1955).

References: 1, Bray *et al.* 1953; 2, Betts *et al.* 1955; 3, Bray *et al.* 1956; 4, Bray *et al.* 1957; 5, Betts *et al.* 1957; 6, this paper.

	Lability of groups			Metabolites excreted in urine							
					Chloroaniline			Glucosiduronic acid			
Position of chlorine atoms	$10^5\ m_{Cl}$ (l. mole^{-1} min.$^{-1}$)	m_{NO_2} (percentage of N liberated as NO_2^-)	Group replaced by acetylcysteine in rabbit, and position	Mercapturic acid (% of dose)	Free (% of dose)	Combined (% of dose)	Chloroazoxybenzene (% of dose)	Naphthoresorcinol method (A) (% of dose)	Copper-reducing method (% of A)	Ethereal sulphate (% of dose)	Ref.
2:3:4:5:6*	—	43	NO_2	37	10	21	—	†	†	0	2
2:3:4:**5***	—	0	Cl, 2	6‡	16	Trace	—	61	100	9	1, 6
2:3:4:6	—	20	NO_2	37	11	20	—	33	94	0	2
2:3:5:6*	—	82	NO_2	36	23	0	—	35	—	3	1
2:3:4	191	0	Cl, 2	41	11	4	—	28	96	11	5
2:3:5	84	14	Cl, 2	21	13	3	—	36	70	11	5
2:3:6	5	8	NO_2	10	19	0	—	67	57	8	5
2:4:5§	729‖	0	Cl, 2	30	21	8	>1	40	81	4	5
2:4:6§	9	4	NO_2	<1	10	22	—	31	60	2	5
3:4:**5**	814	0	Cl, 4	24	24	2	>1	27	73	13	5
2:3	5	—	None	0	15	5	—	47	53	17	6
2:4	50	—	Cl, 2	53	8	3	—	5	62	6	4
2:5	10	—	Cl, 2	27	15	1	—	29	100	14	4
2:6¶	<1	—	None	0	4	Trace	—	45	49	10	6
3:**4**	55	—	Cl, 4	45	17	5	>2	13	100	12	4
3:**5**	—	—	None	0	29	0	>2	70	63	8	6
2	1	—	Cl, 2	7	9	0	—	42	—	24	3
3	—	—	None	0	11	0	—	33	—	18	3
4	4	—	Cl, 4	7	9	4	—	19	—	21	3

* From 33 to 62% of these compounds were unabsorbed. † Determination not reliable, see reference 2.
‡ Rough value based on amount isolated. § These compounds undergo hydroxyldechlorination. ‖ For chlorine atom in position 4.
¶ A small amount of a catechol derivative was detected by paper chromatography.

more conjugated than free trichloroaniline was excreted.

From four members of the series, what are probably products of intermediates in the reduction were identified. 3:4- and 3:5-Dichloro- and 2:4:5- and 3:4:5-trichloro-nitrobenzene yielded the corresponding azoxy compounds. Experiments with the 3:4-dichloronitrobenzene did not provide any evidence that the azoxy compound was an artifact formed in the urine from excreted 3:4-dichlorophenylhydroxylamine.

Chloronitrobenzenes would be expected to form phenols by hydroxylation of either the chloronitrobenzene or the chloroaniline formed from it by reduction. The nitrophenol formed from the former could be reduced to an aminophenol. With the exception of two monochloronitrobenzenes, the phenols formed are those which would result from hydroxylation of the nitro compound in the *meta* position and of the aniline in *ortho* or *para* positions or both. With the *o*- and *m*-chloronitrobenzene, there is evidence of hydroxylation of the nitro-compound in the *ortho* and *para* as well as in the *meta* positions [cf. metabolism of nitrobenzene (Robinson, Smith & Williams, 1951)].

Several halogenobenzenes are excreted by the rabbit in the form of catechol derivatives; with some monohalogenobenzenes, in fact, 20–30 % of a dose may be dihydroxylated in this way (Azouz *et al.* 1953; Azouz, Parke & Williams, 1955; Parke & Williams, 1955). Theoretically catechols could be formed by dihydroxylation of 2:3:4- and 2:3:6-trichloro- and of the dichloro- and the monochloronitrobenzenes. Urines from rabbits given these compounds were examined for catechols by paper chromatography, but only 2:6-dichloronitrobenzene provided evidence of dihydroxylation.

Another possible but unusual route for the formation of phenols was revealed with 2:4:5- and 2:4:6-trichloronitrobenzene. Both these compounds underwent hydroxyldechlorination with the formation of aminodichlorophenols. With 2:4:6-trichloronitrobenzene the aminodichlorophenol was, in fact, the major phenolic metabolite. The same phenol was the major phenolic metabolite of 2:4:6-trichloroaniline. Careful search failed to reveal evidence of this unusual reaction with the other chloronitrobenzenes.

With the 15 members of the series examined, the excretion of copper-reducing material appeared to be related to the excretion of glucuronic acid and in five compounds was quantitatively equivalent; in none did the copper-reducing value of the urine correspond to less than 50 % of the glucuronic acid excreted. A detailed examination of 2:3:6-trichloronitrobenzene showed that the ratio of the glucuronic acid/copper-reducing material was constant in successive urine samples. On the basis of existing evidence, however, it cannot be decided whether the reducing material excreted was labile *O*-glucosiduronic acid, some other form of glucuronic acid conjugate, e.g. an *N*-glucosiduronic acid, or both. Since in many instances the amount of aniline excreted was far less than the amount of glucuronic acid excreted, it is probable that at least some of the glucuronic acid is in the form of labile ether-type glucosiduronic acids. This is supported by the fact that many of the aminophenols were isolated from unhydrolysed urine.

The study of the chloronitrobenzene series has revealed new ways in which, indirectly, conjugation *in vivo* can take place through nuclear substituents. Bray, Ryman & Thorpe (1948) distinguished three types of conjugation reactions according to the nature of the substituent groups involved at the site of conjugation, namely those which could be conjugated directly without modification, e.g. $-CO_2H$, $-OH$, $-NH_2$ (centres for conjugation), those occurring at groups which could be converted *in vivo* into centres for conjugation, e.g.

$$-CH_3 \rightarrow -CO_2H, \quad -NO_2 \rightarrow -NH_2$$

(potential centres of conjugation), and those which were replaced by a new conjugable group before conjugation could occur (formation of new centres for conjugation). At that time the formation of a new centre was envisaged as the replacement of the hydrogen in an unsubstituted position in the ring either by hydroxyl or by cysteyl; the hydroxyl group would then be conjugated with glucuronic or sulphuric acid in the usual way and the cysteyl group with acetic acid to form mercapturic acids, e.g. 1-naphthylmercapturic acid from naphthalene (Bourne & Young, 1934). The chloronitrobenzenes have shown that groups other than hydrogen can be replaced to provide a new centre for conjugation. Such groups may be described as providing 'replacement centres for conjugation'. Both nitro and chloro groups may be replaced by a cysteyl group and the resulting compound can then be conjugated with acetic acid to give a mercapturic acid. A nitro group may thus act as either a potential

$$(-NO_2 \rightarrow -NH_2)$$

or, when labile, a replacement

$$(-NO_2 \rightarrow -S\cdot CH_2\cdot CH(NH_2)\cdot CO_2H)$$

centre for conjugation with acetic acid. Chloro groups may also be replaced by a hydroxyl group and the resulting phenol can be conjugated in the usual way. Thus chloro groups may act as replacement centres in two ways, although in the compounds examined there is no example of chlorine being replaced in the two ways at the same position in the ring, e.g. in 2:4:5-trichloronitrobenzene the mercapturic acid is formed by replacement of

chlorine in position 2 but the aminodichlorophenol by replacement in position 4. The latter metabolite may, however, have been formed from the trichloroaniline rather than the trichloronitrobenzene (cf. 2:4:6-trichloronitrobenzene). Fluoro, bromo and iodo groups have also been shown to act as replacement centres for the formation of mercapturic acid (Bray *et al.* 1957; Betts *et al.* 1957; Bray & James, 1957).

One reason for the examination of all 19 members of the chloromononitrobenzene series was to search for compounds which would be suitable for a study of the mechanism of the formation of mercapturic acid. 2:3:4:6-Tetrachloro-, 3:4:5-trichloro- and 3:4-dichloro-nitrobenzene seem the most suitable for this purpose, and experiments are in progress with these compounds and with benzyl chloride and its nuclear-substituted derivatives in which the side-chain chlorine atom is much more labile than in nuclear chloronitrobenzenes. Preliminary results support the view that glutathione is involved in the process (Barnes & James, 1957; Bray & Franklin, 1957). The effect of the length of the side chain in ω-bromoalkylbenzenes on the formation of mercapturic acid is also being investigated.

SUMMARY

1. The metabolism of 2:3-, 2:6- and 3:5-dichloronitrobenzene has been studied in the rabbit.

2. The main products excreted are phenols, conjugated with glucuronic and sulphuric acids, and dichloroanilines. No mercapturic acids were detected in the urines.

3. Several nitro- and amino-dichlorophenols have been identified. A small amount of a catechol derivative is probably formed from 2:6-dichloronitrobenzene.

4. 3:5:3′:5′-Tetrachloroazoxybenzene has been isolated from the urine of rabbits given 3:5-dichloronitrobenzene.

5. A mercapturic acid, probably *N*-acetyl-*S*-(2:3:4-trichloro-6-nitrophenyl)-L-cysteine, has been isolated from the urine of rabbits given 2:3:4:5-tetrachloronitrobenzene.

6. The chief features of the metabolism of the 19 chloromononitrobenzenes have been summarized and discussed.

We wish to thank Mr P. B. Wood and Mrs B. G. Taylor for assistance with the quantitative analyses. Some preliminary experiments with 3:5-dichloronitrobenzene were performed by Dr Z. Hybs.

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Incorporation of Radioactive Amino Acids into Proteins of the Microsome Fraction of Guinea-Pig Liver in a Cell-Free System

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Although the synthesis of specific soluble proteins has rarely, if ever, been unequivocally demonstrated to occur in cell-free preparations of mammalian cells, the capacity for the uptake of amino acids into peptide linkage in 'non-specific' protein is less readily destroyed by damage to the cell. Thus the incorporation of labelled amino acids into the acid-precipitable protein of tissue homogenates will continue for some time after the destruction of the cell (Winnick, Friedberg & Greenberg, 1948). In liver homogenates, amino acids are incorporated particularly rapidly into the protein of the microsome fraction (Siekevitz, 1952). In this respect the homogenate resembles the intact liver since, in the normal animal given isotope, the initial incorporation is mainly in the microsome fraction (e.g. Hultin, 1950). Zamecnik & Keller (1954) were able to obtain an active cell-free preparation from liver which consisted of only the microsome and soluble cell-sap fractions. In this preparation incorporation of [^{14}C]amino acids into microsome protein would proceed under anaerobic conditions, provided that an adenosine triphosphate-generating system was present. The amino acids incorporated into microsome protein *in vitro* are undoubtedly linked to protein by peptide linkage since labelled peptides can be isolated by partial hydrolysis of microsome protein (Zamecnik, Keller, Littlefield, Hoagland & Loftfield, 1956). It has not, however, been clearly established whether the incorporation of amino acids into protein in such cell-free systems involves the same mechanism as occurs in the intact cell.

The present paper provides some information concerning the difference between the incorporation process in the intact liver cell and in a cell-free system. The technique employed in this study was that of fractionation of the microsome material by successive extraction with solutions of varied ionic strength and pH. By this method a number of subfractions of distinct composition are obtained (Simkin, 1955; Simkin & Work, 1957). We have previously reported the application of this method to a study of the incorporation of [^{14}C]amino acids into the microsome fraction of the liver of the intact guinea pig, and it was established that amino acids were taken up into the proteins of the various subfractions at quite different rates (Simkin & Work, 1957). We have now applied this method to microsome material isolated after incubation with [^{14}C]-amino acids in a cell-free system of guinea-pig liver similar to the type described by Zamecnik & Keller (1954). A preliminary report of this work has been made (Simkin, 1957).

EXPERIMENTAL

Animals. Young guinea pigs from the Institute stock were used (400–500 g., about 4 months old). The animals were starved overnight before use.

Materials. All reagents, except K_2HPO_4 and 3-phosphoglycerate, were of analytical reagent grade. 3-Phosphoglycerate was purchased as the barium salt from C. F. Boehringer und Soehne, Mannheim. A mixture of uniformly ^{14}C-labelled L-amino acids was prepared by acid hydrolysis of [^{14}C]chlorella protein (Radiochemical Centre, Amersham, Bucks). The hydrolysate, after removal of HCl, was adjusted with NaOH to pH 7 before use. All solutions were prepared with deionized water.

Preparation and incubation of the cell-free system. The animals were killed by a blow on the head, and their livers removed and minced as described by Simkin & Work (1957). Liver mince was homogenized with three times its weight of 0·25 M-sucrose for 25 sec., with similar conditions of homogenization to those reported previously. The larger particles were removed from the homogenate by centrifuging twice as before; the resulting preparation is referred to as the microsome–cell-sap fraction (or preparation).

The microsome–cell-sap fraction (10 ml.) was added to 4·4 ml. of a 0·18 M-sucrose solution which contained KCl, 0·0655 M; $KHCO_3$, 0·082 M; K_2HPO_4, 0·045 M; KH_2PO_4, 0·00425 M; $MgCl_2$, 0·0082 M; potassium 3-phosphoglycerate 0·033 M; [^{14}C]chlorella protein hydrolysate, 0·00059 M, 2·71 μc/ml. The reaction mixtures were incubated at 37° with shaking in a vigorous stream of $N_2 + CO_2$ (95:5, v/v). After incubation, 1·5 vol. of an ice-cold 0·25 M-sucrose solution containing unlabelled amino acids (0·002 M with respect to casein hydrolysate N) were added and the mixture cooled in ice. In the experiment in which the specific activity of the acid-soluble fraction of the cell sap was investigated, the sucrose solution added at the end of incubation did not contain unlabelled amino acids.

Preparation of the microsome subfractions and cell sap. The microsome fraction was sedimented without delay by centrifuging at 30000 rev./min. ($g_{av.} = 78000$) for 60 min. in Rotor 30 of a Spinco Model L ultracentrifuge. The supernatant was removed as previously described and taken as the cell-sap fraction. The microsome pellets were washed with 0·25 M-sucrose as before and stored overnight at 2° (when

non-incubated material was fractionated, this was stored if necessary at −10° for several days before fractionation).

Fractionation procedure. When microsome material was fractionated, the material obtained from four separate incubation flasks set up as described above was pooled together for extraction. The pooled material representing the microsome material from about 15 g. of liver was washed twice with 38% (v/v) glycerol as reported previously, but the volume chosen for each washing was about 38 ml. and the centrifugal force employed was 30 000 rev./min. ($g_{av.}$ = 78 000) for 30 min. in Rotor 30 of the Spinco Model L ultracentrifuge.

The fractionation procedure employed was essentially that of Simkin & Work (1957), but certain changes were made. The volume of solution used for the first extraction with either 0·14 M-NaCl or M-NaCl, and extraction with buffer (pH 9) or 0·01 N-NaOH, was about 12 ml. This corresponded to the volume of extractant/mg. of original N previously employed, but in the second extraction with 0·14 M-NaCl or M-NaCl 24 ml. of extractant was used (about two-thirds of the volume used previously). At each stage, the insoluble material was separated by centrifuging for 10 min. at 40 000 rev./min. ($g_{av.}$ = 105 000) in Rotor 40 of the Spinco Model L ultracentrifuge, except at the stage of separation into fractions *D* and *E*, where the time of centrifuging at this speed was reduced to 5 min.

In several experiments involving longer periods of incubation, the non-incubated microsome material was not fractionated, the specific activity of only the whole microsome protein being determined. In these cases, the washing procedure with 38% glycerol was omitted, the microsomes being suspended in iced water before the precipitation of protein.

Isolation of protein for radioactivity measurement and determination of radioactivity. Protein was precipitated with trichloroacetic acid (TCA), reprecipitated, purified and counted as described by Simkin & Work (1957). Under the conditions used 10^{-3} μc/mg. of acrylate resin gives 1000 counts/min. In Table 1 of Simkin & Work (1957) the standard was mistakenly referred to as carbon.

Determination of specific activity of the TCA-soluble fraction of the cell sap. To a sample of the cell-sap fraction at 0°, an equal volume of ice-cold 10% (w/v) TCA was added. The precipitate of protein was centrifuged down in the cold, and the supernatant removed and taken as the TCA-soluble fraction. The TCA-soluble fraction was diluted tenfold with water before determination of specific activity. The radioactivity of the diluted material was determined by evaporating a sample (0·1–0·2 ml.) on a 1 cm.² polythene planchet containing a drop of very dilute detergent (to aid spreading of material over the surface of the planchet). The samples were then counted as described by Simkin & Work (1957). The 'amino N' content of a sample (4·0 ml.) of the diluted material was determined as described below.

Amino nitrogen. This was determined colorimetrically by the β-naphthoquinonesulphonate method of Frame, Russell & Wilhelmi (1943), as modified by Russell (1944). The reagents used were proportionately reduced to give a final total volume of 10 ml. A solution containing equimolar quantities of glycine and glutamic acid was used for determining a standard curve, as suggested by Frame *et al.* (1943). Reagent-blank values were determined by using solutions containing sucrose and TCA at concentrations equivalent to those present in the material assayed. Control experiments showed that such solutions occasionally caused a slight reduction (10% or less) in the colour values given by the standard solution containing glycine and glutamic acid. Duplicate determinations on assay material were in excellent agreement.

Protein and ribonucleic acid. These were estimated by methods similar to those reported by Simkin & Work (1957). The method of separation used was that described for estimation of ribonucleic acid and acid-soluble nucleotide; the protein remaining after extraction with M-$KClO_4$ at room temperature was dissolved in N-NaOH and estimated.

Ultraviolet-absorption curves. Ultraviolet-absorption curves were determined with a Unicam SP. 500 spectrophotometer.

RESULTS

Incorporation of [^{14}C]amino acids into microsome proteins in a cell-free system

The time course of incorporation of a mixture of [^{14}C]amino acids ([^{14}C]chlorella protein hydrolysate) into microsome protein when a microsome–cell-sap preparation of guinea-pig liver was incubated under the conditions described is shown in Fig. 1. Incorporation takes place in an approximately linear manner for about 20 min. Other data indicate that there is no change in specific activity of microsome protein between 20 and 40 min. Similar curves have been reported by Zamecnik & Keller (1954).

Fig. 2 shows the time course of incorporation of [^{14}C]amino acids into the proteins of microsome

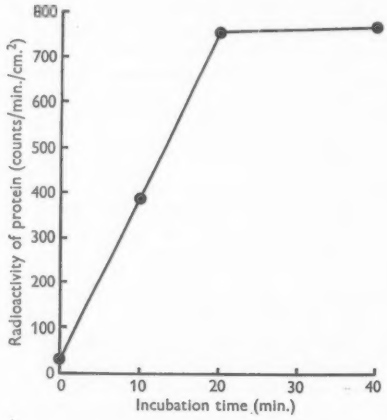

Fig. 1. Incorporation of [^{14}C]amino acids into microsome protein after incubation of a cell-free system prepared from guinea-pig liver. The incubation mixture contained: microsome + cell-sap fractions; KCl (0·020 M); $KHCO_3$ (0·025 M); potassium phosphate buffer, pH 7·8 (0·015 M); $MgCl_2$ (0·0025 M); potassium 3-phosphoglycerate (0·010 M); [^{14}C]chlorella protein hydrolysate (0·00018 M, 0·83 μc/ml.) For other details, see text.

subfractions in a cell-free system. The microsome material was fractionated by the procedure of Simkin & Work (1957) after incubation of the microsome–cell-sap preparations for different times. The preparations were not incubated for longer than 20 min., because linear incorporation continues only for about that time in this system (Fig. 1). A different microsome–cell-sap preparation was used for each time point studied; the 20 min. results are the mean values for two separate experiments (the values were in close agreement). In all experiments, a value for the incorporation of amino acids into the non-incubated microsome material was also obtained. In three experiments (with incubation periods of 10, 15 and 20 min.) the non-incubated microsome material was not fractionated; the values used for correcting for zero time uptake into the microsome subfractions were derived from the mean distribution pattern obtained from three other preparations (the mean zero-time incorporation values for the proteins of the subfractions were as follows, the whole microsome protein value being taken as 1·00: *A*, 0·22; *B*, 0·52; *C*, 0·45; *D*, 0·70; *E*, 1·29). Any error resulting from the use of the zero-time mean values will be small because the specific activity of the non-incubated microsome material was only about 10% of that of the incubated microsomes.

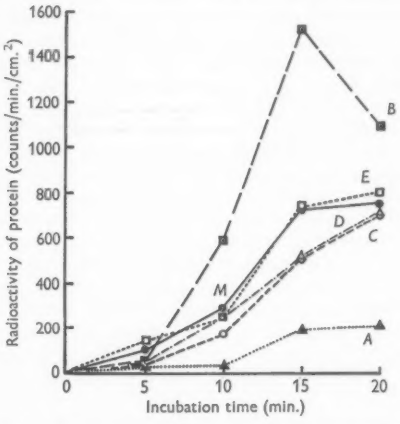

Fig. 2. Incorporation of [^{14}C]amino acids into the protein of microsome subfractions after incubation of a cell-free system from guinea-pig liver. The cell-free system was prepared and incubated as described in the text; after incubation, the microsome material was isolated and fractionated. The values have been corrected for zero-time uptake (see text). The specific activities of the proteins are represented by: whole microsome *M*, ●—●; fraction *A*, ▲···▲; fraction *B*, ■——■; fraction *C*, ○– – –○; fraction *D*, △—·—△; fraction *E*, □·· ·□.

Fig. 2 shows that the protein of microsome subfraction *A* (extracted with 0·14 M-NaCl) had, throughout the whole period studied, a much lower activity than that of the whole microsome protein. The protein of fraction *B*, a ribonucleoprotein subfraction extracted by M-NaCl, had an activity only about one-half that of the whole microsome protein at both zero time and at 5 min.; but there then followed a rapid increase in specific activity, so that at 10 and 15 min. the protein had an activity about twice that of the whole microsome protein, and represented about 25% of the total radioactivity of the whole microsome protein (cf. about 6% at 5 min.). The radioactivity had declined somewhat from its maximum level after 20 min. incubation. In contrast, the protein of fraction *C*, another ribonucleoprotein subfraction isolated by extraction with 0·1 M-bicarbonate–carbonate buffer (pH 9), showed a gradual increase in activity during incubation, the value gradually approaching but not exceeding the whole microsome-protein value. The radioactivity of this fraction represented some 5% of the total after 20 min. incubation. The lipid-containing fractions *D* and *E*, separated by their differing solubility in 0·01 N-NaOH, showed a somewhat different pattern of labelling. The protein of fraction *E* had an activity higher than that of the whole microsome protein at zero time and after 5 min. incubation, whereas the activity of fraction *D* was somewhat lower. Further incubation beyond 5 min. resulted in a gradual increase in the activity of both subfractions, approximately in proportion to the increase in the whole microsome-protein activity, fraction *E* being found to have an activity approximately equal to that of the whole and *D* somewhat lower. At both 0 and 5 min. the proteins of fractions *D* and *E* together accounted for about 90% of the radioactivity of the whole microsome protein, but after 10 and 15 min. incubation this proportion had fallen to 70%.

Significance of incorporation of [^{14}C]*amino acids into microsome proteins in a cell-free system*

The results quoted above, in particular those for the pattern of incorporation of amino acids into the proteins of microsome subfractions *B* and *C*, are quite different from those reported earlier (Simkin & Work, 1957) for the labelling of these subfractions *in vivo* (Fig. 3). It was therefore necessary to establish that the subjection of microsome material to the conditions of incubation did not result in artifacts, such as a redistribution or loss of radioactivity. The following control experiment was therefore carried out. [^{14}C]Chlorella-protein hydrolysate (15 μc/kg. body weight, 3·1 μmoles of N/kg. body weight) was injected into two guinea pigs (mean weight, 510 g.), which were killed 30 min. later, at which time the radioactivity of the protein of

microsome subfraction *C* would be expected to be at or near its maximal level (Simkin & Work, 1957). The microsome–cell-sap fraction was isolated from the pooled livers in the usual way and incubated for 10 min. under the conditions previously described, except that no [^{14}C]amino acids were added to the incubation mixture. The values for incorporation into the proteins of the microsome subfractions subsequently isolated in this experiment are shown in Table 1, together with a comparison of results previously obtained for two other guinea pigs killed 30 min. after injection of labelled amino acids. It will be seen that there is little change in the specific activity of the whole microsome or cell-sap proteins as a result of incubation. The radioactivity of the TCA-soluble material of the cell sap present during the incubation of this sytem was about 0·5 % of that normally present in the complete system *in vitro*. There was in general little change in the distribution of radioactivity between the proteins of the microsome subfractions when compared with the non-incubated values. Such differences as were found are probably due to variations between individual animals; the animals used in the incubation experiment were about half of the weight of those used previously. It would be expected that the smaller animals would have the higher rate of metabolism, and the differences found between the two sets of values are consistent with this supposition. It would thus appear probable that incorporation data obtained in cell-free systems under the conditions described and involving periods of incubation similar to those employed would give a substantially correct measure of the actual pattern of labelling.

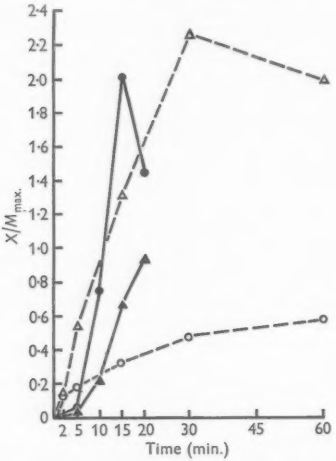

Fig. 3. Comparison of the incorporation of [^{14}C]amino acids into the proteins of microsome subfractions *B* and *C* in a cell-free system prepared from guinea-pig liver with that occurring in the liver of the intact guinea pig. Radioactivity is expressed as the ratio of the specific activity of the subfraction protein (*X*) to that of the highest complete microsome-protein value found in the time considered ($M_{max.}$) (20 min. value for the cell-free system and 60 min. value for the intact animal). Fraction *B in vitro*, ●—●; fraction *C in vitro*, ▲—▲; fraction *B in vivo*, ○— —○; fraction *C in vivo*, △— —△.

Some data have also been obtained relating to the composition of the microsome material isolated from the cell-free systems after varying periods of incubation. The ultraviolet-absorption spectra of the microsome subfractions were determined in all experiments, and the protein and ribonucleic acid (RNA) content of whole microsome material and also of the subfractions was determined in several. It was found that, while there was little significant change in the total quantity of microsome protein after periods of incubation of up to 40 min. duration, there was some loss of microsome RNA, amounting after 30 min. incubation to about 35 % of the RNA present in the zero-time control. As the composition of the subfractions isolated from microsome material incubated for only short periods was comparable to that of the corresponding zero-time control materials, it is probable that the loss of RNA is distributed throughout the various subfractions, as this would result in only a relatively small decrease in the RNA content of each subfraction. Some evidence was obtained suggesting that the RNA of

Table 1. *Distribution of radioactivity among proteins of liver-microsome subfractions obtained from guinea pigs killed* 30 *min. after intravenous injection of* [^{14}C]*amino acids*

In (I), the microsome–cell-sap fraction was incubated, without further addition of [^{14}C]amino acids, for 10 min. under the conditions described in the text. The values in parentheses are those of zero-time controls. (II) represents values found previously (Simkin & Work, 1957) for subfractions labelled *in vivo*: no incubation step was included. The results are expressed as the ratio of the specific activity of the protein isolated to that of the corresponding whole microsome protein. *M* represents the protein of the whole microsome fraction and *S* that of the cell sap; *A*, *B*, *C*, *D* and *E* represent the proteins of the various microsome subfractions.

	M	*S*	*A*	*B*	*C*	*D*	*E*
(I) Incubated	1·00 (0·94)*	0·24 (0·24)	1·54	0·56	1·92	0·88	0·73
(II) Non-incubated	1·00	0·24	1·08	0·50	2·36	0·88	0·70

* Washed with aqueous 38 % (v/v) glycerol.

microsome subfraction *C* is more readily lost than that of subfraction *B*. The ratio of the extinction at 260 mμ to that at 280 mμ of fraction *B* was found to be similar in microsome material isolated after periods of incubation of 20 min. or less; the mean of 11 values was 1·86 ± S.D. 0·15. In fraction *C*, however, while there was little difference in this ratio after incubation for periods of up to 10 or 15 min. (mean of eight values for periods of 10 min. or less: 1·82 ± S.D. 0·12), after 20 min. incubation, lower values (1·39, 1·55) were found, suggesting a lower RNA/protein ratio. The ultraviolet-absorption spectra of subfractions obtained from incubated microsome materials were in other respects similar to those from zero-time materials, thus indicating that the loss of RNA found is probably the major change occurring as a result of incubation, and also that the microsome material is not altered in such a way as to interfere with the fractionation procedure.

In the animals used for the present study, the amount of fraction *C* obtained was relatively somewhat smaller than was found in the animals used for the *in vivo* studies previously reported (Simkin & Work, 1957), fraction *C* representing only about 5% of the whole microsome protein, as compared with some 10–15% previously found. The RNA/protein ratio was, however, of the same order as that previously reported. The microsome fraction in the experiment in which material labelled *in vivo* was incubated (Table 1) had a relatively low content of fraction *C*; the incorporation data obtained do, however, indicate that the pattern of incorporation into the proteins of the microsome subfractions is essentially similar to that reported previously. A relatively low proportion of fraction *C* protein has also been found in other recently fractionated microsome material which had not been subjected to incubation (Bhargava, Simkin & Work, 1958).

Incorporation of [^{14}C]*amino acids into cell-sap proteins in a cell-free system*

It will be seen from Fig. 4 that there is a significant increase in the specific activity of the soluble proteins of the cell sap as a result of incubation of the cell-free system used. There appears to be little increase in specific activity during the first 10 min. of incubation, but a rapid increase in activity then occurs, the extent of the increase being about 10% of the increase in the activity of the whole microsome protein in the same preparation. There is possibly some increase in activity of cell-sap protein after incorporation into whole microsome protein has ceased.

Specific activity of the TCA-soluble fraction of the cell sap

In view of the importance of a knowledge of the specific activity of precursor material for the interpretation of incorporation data, some information on this important parameter was obtained by determining the specific activity of the TCA-soluble material of cell sap isolated after different periods of incubation of the complete incorporation system. The data have been expressed in terms of counts/min./μm-mole of 'amino N'. The colorimetric method of Frame *et al.* (1943) used for the determination of amino nitrogen does not have such a high degree of specificity for amino acids as does

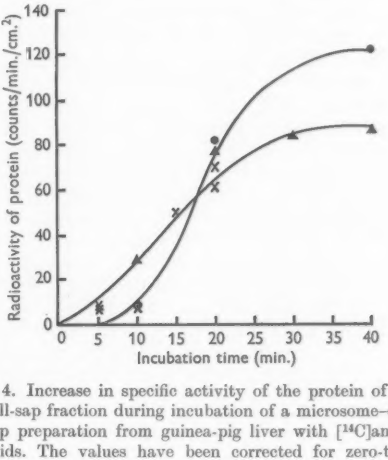

Fig. 4. Increase in specific activity of the protein of the cell-sap fraction during incubation of a microsome–cell-sap preparation from guinea-pig liver with [^{14}C]amino acids. The values have been corrected for zero-time activity. The data shown include two time curves obtained with single microsome–cell sap preparations (●, preparation 1; ▲, preparation 2), as well as values obtained in separate experiments in which each preparation was used for one time point only (×). The cell-sap fractions from preparation 1 were isolated from the same cell-free systems as the microsome material used to obtain the data shown in Fig. 1.

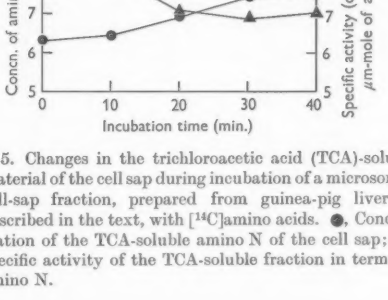

Fig. 5. Changes in the trichloroacetic acid (TCA)-soluble material of the cell sap during incubation of a microsome–cell-sap fraction, prepared from guinea-pig liver as described in the text, with [^{14}C]amino acids. ●, Concentration of the TCA-soluble amino N of the cell sap; ▲, specific activity of the TCA-soluble fraction in terms of amino N.

the ninhydrin–CO_2 method of Van Slyke, Dillon, MacFadyen & Hamilton (1941), but it was considered that the approximate values obtained by the colorimetric method would indicate whether large changes in specific activity were taking place during the period of incubation. It will be seen from Fig. 5 that there is a slight increase in the TCA-soluble amino N level of the cell sap during incubation of the system; as there is little change in the total TCA-soluble radioactivity, this results in a corresponding diminution of specific activity.

Amino acid composition of the proteins of microsome subfractions B and C

Because of the marked differences in the metabolic behaviour of the proteins of microsome subfractions *B* and *C*, and because of their possible importance in the mechanism of amino acid incorporation, an analysis was made of their amino acid composition (Table 2). The analyses were performed by Dr S. Jacobs, using a modified Moore & Stein method (Dr S. Jacobs, unpublished work). It will be seen that there are some differences in the content of a number of amino acids, thus suggesting that the subfractions represent mixtures of different proteins. The relative quantities of the amino acids present are similar to those found in many saline-soluble mammalian proteins (Müting & Wortmann, 1954). The proteins do not appear to be characterized by a high content of basic amino acids (cf. Allfrey, Daly & Mirsky, 1953).

Table 2. *Amino acid composition of the protein of microsome subfractions B and C*

The proteins analysed were the pooled materials isolated for radioactivity determination (Simkin & Work, 1957) in four or five separate experiments. The microsome fractions from which the proteins were isolated had not been subjected to incubation. The proteins were hydrolysed with 6N-HCl, and the amino acids of the hydrolysates separated on columns of Zeo-Karb 225, WR. 1·55 (see text). The results are given in terms of grams of amino acid/100 g. of total amino acid recovered (the tryptophan and cystine contents of the proteins were not determined).

Amino acid	Fraction *B*	Fraction *C*
Aspartic acid	9·5	11·7
Threonine	5·2	3·9
Serine	3·9	5·2
Glutamic acid	12·0	14·9
Proline	7·5	5·7
Glycine	4·7	4·6
Alanine	5·3	5·6
Valine	5·8	6·1
Methionine	2·0	1·8
Isoleucine	4·2	3·9
Leucine	10·2	8·3
Tyrosine	4·0	4·6
Phenylalanine	5·6	7·1
Lysine	9·3	8·1
Histidine	2·5	3·2
Arginine	8·3	5·3

DISCUSSION

The incorporation of labelled amino acids into protein has been used extensively in recent years as a criterion of protein synthesis. While data obtained with the intact animal are unlikely to be invalidated because of artifacts, the results are difficult to interpret because of the complexity of the experimental system, and it thus seemed desirable that protein synthesis should be studied in simpler subcellular systems. When information is obtained from cell-free systems, it is then desirable to compare this if possible with similar information from the intact animal in order to ascertain whether the mechanism of protein synthesis revealed in the subcellular system is in fact similar to that occurring in the intact cell. This poses the problem of how best an experiment can be devised in which data can be obtained relating to protein synthesis in a cell-free system, and in addition enabling such a comparison to be made. Probably the most desirable way in which to carry out such an investigation is to study the synthesis of a specific protein under both sets of conditions. It has, however, in general proved difficult to demonstrate synthesis of specific proteins as measured by either net synthesis or incorporation of labelled amino acids in cell-free preparations (cf. Askonas, Simkin & Work, 1957), although in a few instances some increase in enzymic activity in subcellular systems has been reported, for example, by Gale & Folkes (1955), using disrupted staphylococci, and by Ullmann & Straub (1955), using pancreas. It has not yet been clearly established whether these increases represent protein synthesis. The criterion of protein synthesis in cell-free systems has thus of necessity been the less satisfactory one of incorporation of labelled amino acids into acid-precipitable protein. The present investigation has been designed as an attempt to perform a comparative study of the type outlined above.

The subject of study has been the microsome fraction: this fraction is presumably of importance in the process of protein synthesis in the intact cell, because under these conditions there is a high rate of incorporation of amino acids into microsome protein. Study of this subcellular fraction *in vitro*, where a high turnover rate of protein is also found, might well provide further information on the mechanism of protein synthesis. A comparison has been made under the two sets of conditions between a series of apparently distinct microsome subfractions which are known to have quite varied rates of incorporation in the whole animal (Simkin & Work, 1957).

The results obtained in the present investigation show that in cell-free preparations there is a particularly rapid uptake of labelled amino acids into protein associated with RNA. In this respect we agree

with other workers who have used similar systems from rat liver (Littlefield, Keller, Gross & Zamecnik, 1955; Sachs & Waelsch, 1956). When, however, the incorporation data for the microsome subfractions are compared with those obtained for similar material labelled *in vivo*, it is seen that there are marked differences in the pattern of incorporation into the proteins of the various subfractions. In particular, the difference in behaviour of the ribonucleoprotein fractions *B* and *C* is striking (Fig. 3). In the cell-free system, the protein of fraction *B*, after an initial lag period, shows a very rapid increase in specific activity, whereas that of fraction *C* shows only a gradual increase in activity. In the intact guinea pig, on the other hand, the protein of fraction *C* increased rapidly in activity, whereas fraction *B* had a lower activity than the whole microsome protein during the entire period studied (up to 3 hr. after administration of labelled amino acid). The behaviour of the protein of fraction *A* under the two conditions is also quite different. The proteins of the lipid-containing fractions *D* and *E* do not show marked differences in the pattern of labelling in the two systems. In the cell-free system the protein of fraction *E* has a higher activity than the whole microsome protein during the early stages of incorporation, but this high activity is possibly due to non-specific labelling, perhaps resulting from the technical difficulties encountered in the isolation of protein from such lipoproteins. Thus, while incorporation into protein in a cell-free system resembles that in the corresponding intact cell in that the most rapid uptake of amino acids takes place into protein associated with RNA, the pattern of labelling obtained in cell-free preparations is quite different from that observed in the whole animal. The reasons for these differences are not clear; there are, however, several important differences between the two systems which may play a significant part in determining the altered pattern of incorporation in the cell-free system. The alteration in composition of the microsome fraction which takes place upon incubation may affect the amino acid incorporating system. As mentioned above, evidence has been obtained indicating that there is some loss of microsomal RNA upon incubation, perhaps involving in particular that associated with subfraction *C*. Since it is known that ribonuclease-treated microsomes show a low rate of amino acid incorporation (Allfrey *et al.* 1953; Zamecnik & Keller, 1954), the loss of RNA upon incubation may play a part in determining the pattern of incorporation obtained, and could also explain why incorporation ceases after about 20 min. incubation (cf. Canellakis, 1957). Another important difference between the conditions obtaining *in vivo* and in the cell-free system lies in the level of specific activity of the free amino acid pool during the incorporation process. In the intact animal, the specific activity of the free amino acids of the liver falls rapidly from an initial very high value found almost immediately after intravenous administration of tracer-doses of [^{14}C]amino acid (e.g. Humphrey & Sulitzeanu, 1958), whereas in the cell-free system the specific activity of the free amino acids remains to a first approximation constant (Fig. 5). Thus the cessation of incorporation in the cell-free system, found to occur after 20 min. incubation, is not the result of a fall in the specific activity of the amino acids of the medium.

In the cell-free system studied, after an initial lag period there appears to be some increase in the specific activity of the soluble protein of the cell sap. The extent of the increase found is somewhat larger than that reported in a similar system by Zamecnik & Keller (1954). The increase in the activity of the protein of the cell-sap fraction may represent genuine labelling of the soluble protein present; it will be of interest to ascertain whether specific proteins are labelled in this system. The relatively small increase in the activity of the soluble protein may explain the difficulty in demonstrating synthesis of specific proteins in subcellular systems (see above). The increase in activity of the soluble protein fraction could occur, however, merely as a result of a non-specific loss of labelled microsome protein. Since there is almost four times as much soluble protein as microsome protein in the system used, it may be calculated that the radioactivity of the cell-sap protein after 20 min. incubation is about 40 % of that of the total microsome protein at this time. As no appreciable loss of microsome protein could be detected, a non-specific increase in the activity of the cell-sap protein could be accounted for only by the loss of a small quantity of microsome protein of very high specific activity.

SUMMARY

1. A study has been made of the incorporation of [^{14}C]amino acids into microsome proteins in a cell-free system prepared from guinea-pig liver. The cell-free system, containing microsome and cell-sap fractions, was incubated for varying periods of time with a mixture of [^{14}C]amino acids; after incubation, the microsome material was isolated and fractionated by the extraction procedure of Simkin & Work (1957).

2. In this cell-free system, [^{14}C]amino acids are incorporated into the proteins of the microsome subfractions at quite different rates. The protein of a subfraction containing ribonucleic acid (RNA) (fraction *B*) attained a specific activity more than twice that of the whole microsome protein, whereas the protein of another RNA-containing subfraction (fraction *C*) showed a much slower increase in activity.

3. The data obtained have been compared with the results previously reported from the application of the same fractionation technique to guinea-pig-liver microsome material labelled *in vivo*. The pattern of incorporation into the subfractions found in the cell-free system is quite different from that found in the intact liver cell: in particular, the behaviour of the proteins associated with RNA is markedly different.

4. Changes which occur in the microsome material as a consequence of incubation have been invcstigated: an appreciable loss of RNA was found to occur. The significance of the incorporation of [^{14}C]amino acids into microsome protein in this cell-free system has been discussed.

5. A brief study was also made of the changes in the specific activity of both the soluble protein and of the trichloroacetic acid-soluble material of the cell-sap fraction after incubation of this cell-free system with [^{14}C]amino acids.

We wish to thank Miss A. Kraty for valuable technical assistance, and Dr S. Jacobs for carrying out the analyses of amino acids.

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Polyphosphates Excreted by Wax-Moth Larvae (*Galleria mellonella* L. and *Achroia grisella* Fabr.)

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During their studies on the biochemistry of *Galleria mellonella*, the greater wax-moth, Niemierko (1950) and Niemierko & Niemierko (1950*a*, *b*) observed that these organisms excreted large amounts of an acid-labile phosphate which was not present in their food. They identified this material as inorganic polyphosphate, and showed that 85% of the total orthophosphate consumed was excreted in this form. Polyphosphates are also formed by *Achroia grisella*, the lesser wax-moth, but not by a number of other insects, suggesting that the formation of these compounds is connected with the ability to digest and assimilate wax.

Although the polyphosphates are widespread in bacteria, moulds and algae (Schmidt, 1951), Niemierko's work is, so far, the only convincing identification of these compounds in material derived from higher animals. Niemierko & Niemierko (1950*a*, *b*) present some evidence that they were not produced by intestinal bacteria. It has been claimed that small amounts of polyphosphates occur in the fat bodies of other insects (Heller, Karpiak & Zubikowa, 1950), but details of the identification were not given. As large amounts of the excreta of both wax-moths were available, it seemed worth while to confirm the results of Niemierko & Niemierko and to attempt to identify the polyphosphates present by ion-exchange chromatography.

EXPERIMENTAL

The excreta of both moths were provided by Dr L. Bailey. The moths were cultured at either 35° (*G. mellonella*) or room temperature (*A. grisella*) in large glass jars containing

Table 1. *Barium precipitation of acid-labile phosphates*

To 2 ml. samples containing 60–200 μg. of P was added 3N-acetate buffer (2 ml.) and saturated $Ba(NO_3)_2$ (1 ml.). The mixture stood on ice overnight and was then centrifuged at 2000 *g* for 15 min. The precipitate was dissolved in N-HCl and the acid-labile phosphate estimated after the barium had been removed as sulphate. The pH of the supernatant solution was measured by means of a glass electrode.

	Per cent precipitated			
pH	Acid-labile phosphate in extract of excreta of *G. mellonella*	Tripolyphosphate	Trimetaphosphate	Tetrametaphosphate
3·5	0	0	0	0
3·8	14	—	—	—
4·0	—	45	0	0
4·1	30	—	—	—
4·5	—	70	0	0
4·7	50	77	1	0
5·1	74	90	2	0
5·5	—	—	4	0
5·9	92	—	—	—

pieces of brood-comb. After about 2 months, when the brood-comb had almost disappeared, the moths and larvae were removed. The detritus left in the bottles was a dry mixture of insect excreta, unconsumed brood-comb and cocoons. The latter two materials could be distinguished by eye and were removed as far as possible, leaving a black granular material, the bulk of which was excreta.

Orthophosphate and total phosphate were estimated by the modification of the method of Kuttner & Lichtenstein described by Holden & Pirie (1955). Acid-labile phosphate was estimated as orthophosphate after being heated in N-HCl for 10 min. at 100°.

Chromatography of polyphosphates was carried out on Dowex 1 (100–200 mesh, 8 % cross-linkage) in the manner described by Peters & Rieman (1956). The eluents used in this method were: (1) 0·25M-KCl, (2) 0·40M-KCl, (3) 0·50M-KCl, all buffered at pH 5·0 with 0·005M-acetate buffer; and (4) 3·0N-HCl, which was allowed to stand overnight in contact with the resin in order to hydrolyse any tenaciously absorbed polyphosphates.

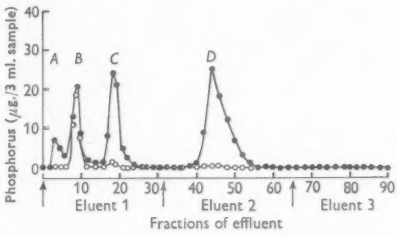

Fig. 1. Chromatography of extracts of the excreta of *G. mellonella*. An aqueous extract (5 ml.; 900 μg. of total phosphorus) was adjusted to pH 5·0 and chromatographed on a Dowex 1 column. Fractions of the effluent (10 ml.) were collected, and samples (3 ml.) taken for total phosphate (●) and orthophosphate (○) estimations.

RESULTS

Phosphorus accounts for 0·6–0·7 % of the dry weight of the excreta of *G. mellonella*. Repeated extractions with water or 5 % trichloroacetic acid solution removed only about 68 % of this total, and the residue could not be dissolved even after heating for 15 min. on a boiling-water bath with either N-sodium hydroxide or N-hydrochloric acid. About 10 % of the soluble fraction was orthophosphate and 73 % was hydrolysed to this form after boiling for 10 min. with N-hydrochloric acid. The excreta of *A. grisella* contained about 1·3 times as much extractable phosphate as that of *G. mellonella*, but a larger proportion of it (30–40 %) was orthophosphate.

The acid-labile phosphate in the extracts could be precipitated by barium salts (Table 1) under conditions similar to those described by Wiame (1949). Although these results varied a little from experiment to experiment, probably according to the concentration of the precipitable phosphate, there was never any precipitation below pH 3·5, and it neared completion only at about pH 5·5. This suggests the behaviour of low- rather than high-molecular-weight polyphosphates (Krishnan & Damle, 1956). Tripolyphosphate behaved in a similar manner (Table 1), whereas the cyclic tri- and tetra-metaphosphates were not precipitated at all under these conditions.

Lead is a more effective precipitant than barium, and nearly all the acid-labile phosphate in the extracts was precipitated at pH 3 by lead acetate.

Aqueous extracts of the excreta could be chromatographed successfully on Dowex 1 columns, without any preliminary purification. The capacity of the resin for the phosphate in this solution was, however, small, and less than 1 mg. of total phosphate could be applied. Fig. 1 shows the result of a typical experiment on the material from *G. mellonella*. The phosphate has been resolved into

four components: one is orthophosphate (*B*); two are acid-labile phosphates (*C* and *D*), whose positions suggest that they are pyrophosphate and tripolyphosphate respectively; and one (*A*) is an unidentified acid-stable phosphate. In addition to these, a small amount of phosphate was tenaciously absorbed by the resin and was eluted only on treating the column with 3N-hydrochloric acid as described above. This material may consist of polyphosphates containing more than five phosphorus groups (Peters & Rieman, 1956).

Phosphate recoveries from the column were quantitative (90–100 %). The approximate amounts of the various phosphates present were: component *A*, 3 %; component *B*, 14 %; component *C*, 23 %; component *D*, 44 %; fraction retained by the resin, 16 %. Extracts of *A. grisella* contained the same phosphates in similar amounts, but with somewhat more orthophosphate and less of the material retained on the resin. The proportions were: *A*, 2 %; *B*, 29 %; *C*, 23 %; *D*, 40 %; residue, 6 %. The bulk of the water-soluble phosphates present in both cases (60–70 %) were therefore small-chain linear polyphosphates. No cyclic metaphosphates were detected.

The identification of components *C* and *D* as pyrophosphate and tripolyphosphate respectively is based mainly on their behaviour on the chromatogram, and their quantitative conversion into orthophosphate on boiling with N-hydrochloric acid for 10 min. They were both also quantitatively converted into orthophosphate by a purified preparation of an unspecific pea-phosphatase (Pierpoint, 1957). Although adenosine diphosphate and adenosine triphosphate behave in a similar manner to *C* and *D* during the chromatography they can clearly be distinguished by their absorption at 260 mμ. If all the absorption at this wavelength associated with *C* and *D* were due to the presence of adenosine derivatives, it could not account for more than a few per cent of the total phosphate present in these components.

Measurement of the absorption at 260 mμ of the fractions of eluate revealed the presence of two peaks of absorbing materials. The first was in fractions 4–7, and corresponded to material that had been washed straight through the column. These tubes contained the bulk of the brown coloration that was present in the initial extracts, and their ultraviolet-absorption spectrum did not have any distinct maxima. The second peak was in fractions 23–29. Its ultraviolet-absorption spectrum had maxima at 235 and 290 mμ and closely resembled that recorded for uric acid (Johnson, 1951). The ratio of absorption at these two wavelengths was 0·87, while that for a commercial sample of uric acid dissolved in eluent 1 was 0·90.

DISCUSSION

The present work confirms the excretion of polyphosphates by the wax-moth larvae, and suggests that the bulk of them are small straight-chain compounds. It throws no light on the possible role of the intestinal bacteria in the formation and degradation of these compounds. It is possible that the particular phosphates observed were the result of a bacterial breakdown of high-molecular-weight polyphosphates which took place in the moths' intestines. It is less likely that they were the result of such a breakdown taking place after the excreta were voided, because of the extreme dryness of the excreta. They contain only 1 or 2 % of water and thus make an unfavourable medium for enzyme action or bacterial growth. The similarity in phosphate composition of the excreta of the two insects also argues against this latter breakdown; moreover, phosphatase activity could not be detected in aqueous extracts of the excreta.

Recently Wojtczak (1956) has analysed the phosphates excreted by starving larvae of *G. mellonella*, with results somewhat different from those described here. About half the acid-labile phosphate present was pyrophosphate, and the rest consisted mainly of a more highly polymerized compound which was hydrolysed to trimetaphosphate during paper chromatography in acid solvents. This polyphosphate gave a metachromatic effect with toluidine blue. No such metachromatic effect could be observed with the smaller amounts of highly polymerized polyphosphates detected in the present work, but this may have been due to masking by the brood-comb pigments present in the excreta of non-starving wax-moths.

There have been reports that the cells of the ventriculus of the honeybee contain a large number of phosphate-rich granules (Bailey, 1955; Day, 1949). An attempt was therefore made to detect polyphosphates in trichloroacetic extracts prepared from the mid-gut of 243 honeybees. Large amounts of orthophosphate and an acid-stable phosphate that emerged from the column before the orthophosphate were observed, but no polyphosphates. If they were present at all they must have accounted for less than 2 % of the total phosphate.

SUMMARY

1. The excreta of wax-moth larvae (*Galleria mellonella* L. and *Achroia grisella* Fabr.) feeding on brood-comb contains approximately 0·5–1·0 % of its dry weight as phosphorus. About half of this is present as water-soluble acid-labile phosphates, and about a third is not extracted with water, boiling acid or boiling alkali.

2. The composition of the water-soluble phosphates excreted by *G. mellonella* was found by

anion-exchange chromatography to be orthophosphate (14 %), acid-stable phosphate (3 %), pyrophosphate (23 %), tripolyphosphate (44 %), and a fraction retained by the resin (16 %). This last fraction probably consists of more highly condensed polyphosphates.

3. The composition of the water-soluble phosphates excreted by *A. grisella* was orthophosphate (29 %), pyrophosphate (23 %), tripolyphosphate (40 %), acid-stable phosphate (2 %), and the fraction retained by the resin (6 %).

I am extremely grateful to Dr L. Bailey of the Bee Department for his kindness in supplying the moth excreta and the bees used in this work.

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A Method for the Determination of Non-Haem Iron in Bone Marrow

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There are many reports in the literature (Rath & Finch, 1948; Masshoff & Gruner, 1951; Hutchison, 1953) of investigations into the iron content of bone marrow from human subjects by various histological techniques. Most of the work has been done with smear preparations or sections of fixed tissue from material obtained at biopsy and stained with acidified potassium ferrocyanide. A deposit of ferri-ferrocyanide (Prussian blue) indicates the presence of iron-containing material. It is well recognized that only haemosiderin will be detected in this way, the Prussian blue being formed on the surface of the haemosiderin particles.

Granick (1954) originally postulated that iron was utilized in the synthesis of haemoglobin only from ferritin and that haemosiderin represented deposits of iron in excess of metabolic requirements. In these circumstances the presence of haemosiderin would imply the presence of adequate amounts of iron and the Prussian blue test could be used as a rough estimate of the body stores of iron. Shoden, Gabrio & Finch (1953), however, have found a simultaneous labelling of both ferritin and haemosiderin after administration of radioactive iron. Similarly, during haemopoiesis they found that iron from both ferritin and haemosiderin was utilized. These findings would suggest the necessity of estimating the total quantity of iron in the bone marrow rather than that fraction present as haemosiderin.

Cavayé Hazen (1949) and others have reported the results of the estimation of iron in bone marrow obtained at necropsy in a variety of diseases. Relatively large amounts of tissue were required for the analysis which was performed on a wet ash of the material.

As it was desirable to use biopsy material it was decided to develop a method requiring only a small quantity of marrow tissue. It has been shown by Krause (1943) and by McCoy & Schultze (1944) that the lipid content of marrow tissue is variable. Dietz (1949) has studied the relationship between nitrogen, lipid and water content in bone marrow from several species. From these results it would appear that any analysis based on tissue weight, either wet or dry, would be unreliable. Davidson, Leslie & White (1947) have stated that with moderate quantities of marrow tissue (30–60 mg.) the values for deoxyribonucleic acid are the least satisfactory of their results, the amount of phosphorus being too small for accurate analysis. It was therefore decided to try to relate the iron content of the marrow to the protein content and this has proved satisfactory. The method for the

measurement of protein is relatively specific, is simple to use and was designed especially for the estimation of small amounts of protein.

A description of the method is given, together with a comparison of the results obtained by this method and by the classical Prussian-blue staining technique.

The results of the measurement of the iron content of bone marrow obtained from a group of hospital patients with no signs of anaemia, from patients with the anaemia associated with rheumatoid arthritis, with iron-deficiency anaemia and with pernicious anaemia are presented.

Results have been expressed as μg. of iron/100 mg. of protein.

METHODS

All apparatus was rendered free from iron by cleaning in 50% HCl and in water after the usual chromic acid treatment. The reagents were frequently checked for iron content.

Preparation of marrow samples

The aspirate, obtained by the technique described by Davidson (1941), was placed in a tube containing heparin as anticoagulant. Marrow particles were transferred by means of a pointed glass rod to a second tube containing 1 ml. of 0·9% (w/v) NaCl. After standing for about 1 hr. at 4° the particles were transferred to an all-glass homogenizer (Potter & Elvehjem, 1936) containing 1 ml. of water and ground to an even suspension. The homogenizer was rinsed with 0·5 ml. of water and this was added to the suspension. The quantity of tissue used was determined by the size of the sample, but 5–10 mg. wet wt. was found to be a convenient amount.

As the quantity of iron to be estimated was very small ($<1\,\mu$g.) a blank solution was prepared by the same procedure but with the marrow omitted.

Samples of both blank and marrow preparations were taken for the estimation of iron and of protein.

Iron estimation

The method was essentially that of Ramsay & Campbell (1954) suitably modified for the small amounts of tissue available.

Reagents. Sodium sulphite. 0·5M-soln. Dipyridyl reagent: 1% (w/v) $\alpha\alpha'$-dipyridyl in 10% (v/v) acetic acid. All reagents were saturated with CO before use.

Method. To 1 ml. of the suspension were added 0·5 ml. of Na_2SO_3 and 1·5 ml. of dipyridyl reagent. The solutions were mixed and heated in a boiling-water bath for 30 min., after which the volume was made up to 3 ml. with water. The solution was cooled and centrifuged at 4°. The intensity of the pink colour in the supernatant was measured against the blank in the Unicam SP. 600 spectrophotometer at 520 mμ.

Protein estimation

The method of Lowry, Rosebrough, Farr & Randall (1951) was used with suitable modification.

Reagents. Alkaline copper reagent: 1 ml. of 0·5%(w/v) $CuSO_4,5H_2O$ in 1% (w/v) sodium potassium tartrate added to 50 ml. of 2% (w/v) Na_2CO_3 (anhydrous) in 0·1N-NaOH. Folin and Ciocalteu reagent (British Drug Houses Ltd.) diluted to 0·1N-acid. Both reagents were prepared freshly before use.

Method. To 0·2 ml. of the suspension was added 2 ml. of the alkaline copper reagent and the mixture allowed to stand at room temperature for 10 min. A volume (1 ml.) of the Folin reagent was added rapidly with mixing and the solution again left at room temperature for 20 min. After centrifuging at 4° the blue colour in the supernatant was measured against the blank in the Unicam SP. 600 spectrophotometer at 750 mμ.

RESULTS

Reproducibility of the method. Several marrow samples were combined and six replicate estimations carried out. The average value for the iron content was found to be 0·45 μg. with a standard deviation of 0·08 μg., and that for the protein $18{\cdot}2 \pm 1{\cdot}4\,\mu$g.

Error of sampling. This was tested in two ways. Particles from an aspirate of marrow were divided into four parts and the iron content of each part was estimated. The average value was 85 μg./100 mg. of protein with a range of 81–90 μg. The iron content was determined in multiple aspirates of marrow from the sternum (three samples) and the iliac crests (two samples) obtained at necropsy from each of two individuals. The average value in the first patient was 42 μg. of iron/100 mg. of protein with a range of 31–53 μg. In the second patient the average was 219 μg. of iron with a range of 197–240 μg.

Comparison of results obtained by the quantitative and histological methods. A quantitative estimate of the iron content was made in the bone marrow from forty-nine individuals and sections of fixed tissue were stained by the Prussian blue method. For ease of comparison the results from the histological method have been grouped into those showing little or no detectable staining (group 1) and those showing definite staining (group 2). The iron content could then be compared in the two groups (Fig. 1). The mean value for group 1 was 45 μg. of iron/100 mg. of protein, with a standard deviation of 27 μg. and, for group 2, $72 \pm 35\,\mu$g. The difference between these values is highly significant ($P < 0{\cdot}01$).

Iron content of bone marrow. The iron content was estimated in marrow from sixteen patients in an Orthopaedic Ward, none of whom showed any signs of anaemia; from fifty-three patients suffering from the anaemia associated with rheumatoid arthritis; from six patients with iron-deficiency anaemia, and from three with pernicious anaemia. The results are shown in Table 1. Analysis of these results has shown that although the average iron content of the marrow in rheumatoid arthritis is reduced as compared with that of the non-anaemic control group, it does not reach the low level found

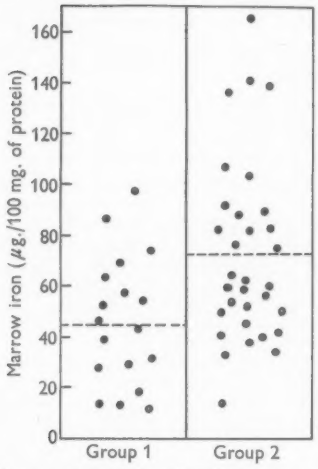

Fig. 1. A comparison of the values for the iron content of bone marrow as determined by the quantitative and Prussian blue methods. Group 1, little or no Prussian blue staining; group 2, definite Prussian blue staining.

Table 1. *Iron content of bone marrow in various anaemias*

Standard deviation and the number of observations (in parentheses) are also given.

Diagnosis	Iron content (μg./100 mg. of protein)
Control, non-anaemic	86±34 (16)
Rheumatoid arthritis	55±30 (53)
Iron-deficiency anaemia	12±5 (6)
Pernicious anaemia	86–136 (3)

in iron-deficiency anaemia. The difference between the average values in the two groups is highly significant ($P = 0{\cdot}01$).

DISCUSSION

Great advances have been made in the study of iron metabolism since the introduction of radioactive isotopes. From the studies of the interrelationship of ferritin and haemosiderin it would appear that both compounds are metabolically active. In view of this it is of importance, especially in bone marrow, to be able to measure the total non-haem iron when assessing the iron content of the tissue. Stress has been laid by Hutchison (1953) on the presence or absence of iron stainable by ferrocyanide in the diagnosis and treatment of anaemia. He claims that only in those patients whose marrow contains no haemosiderin will administration of iron increase haemopoiesis. Beutler, Drennan & Block (1954), however, have shown that a marked improvement in the haemoglobin level can occur following oral-iron therapy without the appearance of haemosiderin in the marrow. Only after prolonged administration of iron had increased the body stores of iron was haemosiderin detectable in the marrow. Thus although a negative Prussian blue test may indicate subnormal amounts of iron in the marrow, it gives no measure of the severity of the deficiency.

The method described above for the estimation of iron in small samples of bone marrow is simple and appears to be reliable. Replicate analysis of single and multiple samples has shown the accuracy to be sufficient for diagnostic purposes. It might be argued that protein is not a suitable constituent of the marrow on which to base the iron content and that deoxyribonucleic acid would give more reliable information in relation to the cellularity of the tissue. These considerations are outweighed by the accuracy of the method for the estimation of protein compared with that for phosphorus at the level encountered in this work. The use of the specific ultraviolet absorption for the estimation of both protein and deoxyribonucleic acid, as recently reported in a review of bone-marrow metabolism (Lajtha, 1957), might lead to an interesting comparison of results.

A good overall agreement between the quantitative and Prussian blue methods can be shown by dividing the results into two groups, those giving definite staining and those giving little or no staining. When individual results are considered, however, it will be seen from Fig. 1 that a marked disagreement may occur. It should be borne in mind that whereas the histological method is confined to the demonstration of iron in the form of haemosiderin, the quantitative method measures the total non-haem iron. Thus considerable disagreement may occur between individual results as estimated by the two methods.

The range of values obtained for the iron content of the marrow from non-anaemic subjects, from patients with pernicious anaemia and iron-deficiency anaemia is in agreement with that generally reported with the Prussian blue method (Rath & Finch, 1948; Davidson & Jennison, 1952). Richmond, Gardner, Roy & Duthie (1956) have studied the iron content of bone marrow from patients suffering from the anaemia associated with rheumatoid arthritis. Although no other sign of iron deficiency was found, 33% of the marrows examined contained no iron detectable by the Prussian blue test. The quantitative method now described was used in a small proportion of these cases, but no normal material was then available for comparison. The results now reported confirm the earlier findings that there is significantly less iron in the marrow in rheumatoid arthritis than is

normally present, but it has also been possible to show that only a minor degree of iron deficiency occurs and only in the rare case does the marrow-iron level reach the low values found in iron-deficiency anaemia. This method may therefore be of use in differentiating between degrees of iron deficiency.

SUMMARY

1. A simple method for the determination of the non-haem iron content of bone marrow is described which is of sufficient accuracy for diagnostic purposes.

2. Results obtained by this method have been compared with those obtained by the Prussian-blue staining method.

3. The non-haem iron content of bone marrow from both non-anaemic and anaemic subjects has been determined.

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The Lipids of Ram Spermatozoa

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Since the pioneering studies of Miescher (1878,1897) it has been known that spermatozoa contain a considerable amount of intracellular lipid. Miescher believed the lipid material, which he extracted from salmon spermatozoa, to consist of 50% lecithin, 35% neutral fat and 14% cholesterol. Studies by Mathews (1897) and Sano (1922) on the semen of various fishes also indicated the presence of a large amount of lecithin in the sperm cells. The lipids of sea-urchin sperm have been analysed by Cardin & Meara (1953) and found to consist of 26·0% phospholipids, 32·9% free fatty acids, 13·6% neutral fat, 9·2% sterols and 18·3% of other unsaponifiable material. The lipid content of bull sperm was first determined by Kölliker (1856), who showed that ether-extractable material accounted for 12% of the dry weight of the sperm. Miescher (1878), who performed the first analysis of the lipid extracted from bull sperm, thought that about half of it consisted of lecithin. More recently, determinations of total lipid and phospholipids in bull sperm were carried out by Lardy & Phillips (1941) and Zittle & O'Dell (1941). The latter authors found that 13% of the dry weight of bull sperm was lipid, the sperm heads containing 7% lipid, the middle pieces 6% and the tails 23%.

The suggestion that lipids occur in spermatozoa in a bound state, presumably in the form of lipoproteins, was made by Halpern (1945) on the basis of his experiments with salmon semen. Mayer and

his colleagues (Mayer & Thomas, 1947; Thomas & Mayer, 1949; Dallam & Thomas, 1953; Mayer, 1955) obtained from the sperm heads of various mammalian species (including bull, boar, ram, dog and man) an alkali-soluble but acid-insoluble protein complex which contained nearly 30% of ether-extractable material composed largely of phospholipid and cholesterol.

The first indication that sperm lipids may be composed, in part, of acetal phospholipids (plasmalogens) came from a study of Feulgen & Rossenbeck (1924), who demonstrated that when fresh smears of semen were treated with Schiff's fuchsin–sulphurous acid reagent, the middle pieces and tails, although devoid of nucleic acid, stained very strongly. Later it was shown that the plasmalogen content of bull semen, as determined by Feulgen's method, varied from 30 to 90 mg./100 ml., and that about two-thirds of this amount was present in spermatozoa with the rest in the seminal plasma (Boguth, 1952).

In view of the scanty knowledge concerning the chemical nature of the lipids present in mammalian spermatozoa, a study was made of the various compounds which make up the intracellular lipid material in the spermatozoa of the ram. This species was chosen on account of the exceptionally high sperm density of semen, and the ease with which ram spermatozoa can be separated and washed free from seminal plasma.

EXPERIMENTAL

Spermatozoa, obtained from freshly collected ram semen by centrifuging and washing (Mann, 1946), were freeze-dried at −20°, powdered and preserved until extraction at −20° in a desiccator. Two batches of washed and freeze-dried ram sperm were available, which were collected during the 1954–55 and 1955–56 breeding seasons and subsequently called 'old sperm' (OS) and 'new sperm' (NS) respectively. Yields, 27·9 g. of OS and 23·3 g. of NS.

Each lot of NS had been kept out of contact with air in a sealed ampoule, and the total product was quite white. No precautions against oxidation had been taken with the OS, which was brown. The OS was examined in one batch, the NS in two batches of 11·0 and 12·3 g. respectively. The lipids were extracted by refluxing with three successive batches (350 ml. each) of $CHCl_3$–methanol (2:1, v/v) and purified by the procedure of Folch, Ascoli, Lees, Meath & Le Baron (1951). They were then further purified by passage in $CHCl_3$–ethanol–water (80:20:2·5, by vol.) through a cellulose column (50 g. of adsorbent; Lea & Rhodes, 1953). The yields of purified lipid were 2·62 g. (9·4%) from the OS (OSL) and 0·83 g. (7·5%) and 0·86 g. (7·0%) from the two batches of NS (NSL 1 and 2 respectively). The extraction and purification scheme is illustrated in Fig. 1.

Since the OSL might have undergone some deterioration during the lengthy storage of the dried spermatozoa, it was decided to use this material for preliminary studies and the NSL for subsequent, more elaborate, fractionation.

Analytical methods

Nitrogen was determined by a micro-Kjeldahl procedure, digestion being with 4 ml. of H_2SO_4, 4 g. of K_2SO_4 and 1 drop of Hg. After digestion for 20 min. the mixture was cooled and 1 ml. of 30% H_2O_2 added. This addition was repeated after a further 20 min. digestion and then followed a final digestion period of 4 hr. Phosphorus was determined by Allen's (1940) method, except that the $HClO_4$ digestion was performed in an oven at 170° for 4 hr. followed by 20 min. at 100° after dilution, to hydrolyse any pyrophosphate that might have been formed. Glycerophosphate was determined according to Olley & Blewett (1950) and choline either as the reineckate (Lovern, 1952) or by paper chromatography (Olley, 1956*b*). Paper chromatography was also employed to determine glycerol (Olley, 1956*a*), inositol and sugar (Olley, 1956*b*). For sugar, hydrolysis was

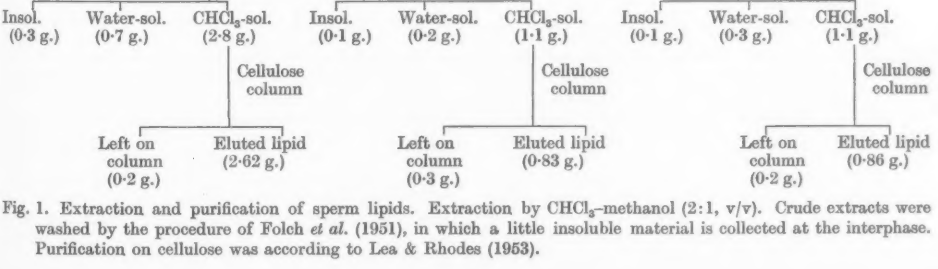

Fig. 1. Extraction and purification of sperm lipids. Extraction by $CHCl_3$–methanol (2:1, v/v). Crude extracts were washed by the procedure of Folch *et al.* (1951), in which a little insoluble material is collected at the interphase. Purification on cellulose was according to Lea & Rhodes (1953).

with 3N-H_2SO_4, as recommended by Mallov, McKibbin & Robb (1953) for cerebrosides; Amberlite IR 4B resin was used and the galactose control put through the entire procedure. Phosphorylcholine was determined by paper electrophoresis (Olley, 1956*b*), with borate buffer at pH 9. In all the above determinations on paper, areas were measured by cutting out and weighing the spots. Ethanolamine and serine were determined colorimetrically after two-dimensional chromatography on Whatman no. 1 paper, phenol–aq. NH_3 soln. and collidine–lutidine–water (Dent, 1948) being used. The air-dried chromatograms were sprayed on both sides with 1% (w/v) ninhydrin in wet butanol, the colour being allowed to develop over 2 days at room temperature (Thompson, 1951). The spots, including the control spots, were then cut out and eluted with 50% (v/v) aq. acetone for colorimetric assay. Sphingosine was determined by the method of McKibbin & Taylor (1949) and cholesterol, free and combined, as by Lovern & Olley (1953*a*).

Acetal phosphatides (plasmalogens) were determined in three ways: (*a*) colorimetrically on the original lipid, (*b*) colorimetrically on the isolated dimethylacetals and (*c*) gravimetrically. No entirely satisfactory assay procedure seems to have been evolved and controversy continues over the colorimetric methods, e.g. Feulgen, Boguth & Andresen (1953); Christl (1953); Leupold, Büttner & Ranniger (1953); Thiele (1955). We have found that whereas the colorimetric method of Feulgen & Grünberg (1938), with $CHCl_3$ as extractant (Lovern, 1952) gave poor replication, better replication and a satisfactory calibration curve (with palmitaldehyde) could be obtained by addition of extra acetic acid. This gave sharp separation of a clear $CHCl_3$ layer. In the procedure finally adopted, 5 ml. of the Feulgen & Grünberg reagent and 2 drops of 6% $HgCl_2$ were added to the samples dissolved in 1 ml. of acetic acid in well-stoppered 20 ml. test tubes. Colour development proceeded overnight at room temperature, after which 8 ml. of a mixture of $CHCl_3$, previously washed three times with water, and acetic acid (1:1, v/v) were added to each tube; the mixture was stirred and left for 10 min. A sample of the lower layer was then withdrawn for colorimetric assay. The more cumbersome procedure of Ehrlich, Taylor & Waelsch (1948) was also used, but only on OSL. Dimethylacetals were prepared according to Klenk & Friedrichs (1952) but, since this product must contain any unsaponifiable matter present in the original lipid fraction, it could not be used for a gravimetric assay. The latter was performed by direct isolation and weighing of the free aldehydes by a method (cf. Olley & Lovern, 1953) in which other unsaponifiable material is extracted before the aldehydes are liberated from the water-soluble alkali plasmalogenates (see below).

Examination of 'old-sperm lipid'

Analyses of N, P, cholesterol and plasmals were carried out on the intact lipid but the main bulk of this material was hydrolysed by two methods, namely, refluxing for 2 hr. with ethanolic 0·5N-KOH (1·05 g. of lipid) or refluxing for 6 hr. with dry methanolic 6N-HCl (1·05 g. of lipid), about 20 ml. of reagent being used in each. In addition, a smaller sample (0·262 g.) was hydrolysed by the special procedure of McKibbin & Taylor (1949) for sphingosine estimation, except that after adding the HCl the mixture was shaken with ether and 10 ml. of the aqueous layer removed for examination. The ether was then boiled off and the acid hydrolysis completed as usual. This permitted examination of the water-soluble products of hydrolysis with aqueous baryta.

After addition of water to the KOH hydrolysate, unsaponifiable matter was extracted with ether. The solution was then acidified with HCl and the mixture of fatty acids and free plasmals was recovered by further extractions with ether. These two products were separated by washing the ethereal extract with aqueous alkali (cf. Olley & Lovern, 1953, and the gravimetric aldehyde assay above). The acidified aqueous phase of the original KOH hydrolysate was evaporated on a steam bath under vacuum, for determination of water-soluble products.

The treatment of the methanolic HCl hydrolysate was a simplification of the procedure of Klenk & Friedrichs (1952). The mixture was extracted by countercurrent distribution in four separating funnels between light petroleum (b.p. 40–60°) and methanolic 6N-HCl. The combined petroleum phases, containing methyl esters of fatty acids, dimethylacetals of the plasmals and any other unsaponifiable matter, were neutralized with solid Na_2CO_3, evaporated and the residue was hydrolysed by refluxing with ethanolic 0·5N-KOH for 2 hr. Addition of water and extraction with ether gave acetals plus other unsaponifiable matter, the fatty acids being later recovered from the aqueous soap layer. The combined methanolic HCl phases were also evaporated. The residue was not entirely water-soluble, but the water-insoluble part was found to be soluble in $CHCl_3$.

It was assumed at this stage that the $CHCl_3$-soluble, water-insoluble material might be sphingosine hydrochloride or other sphingosine-containing derivatives, but that fatty acids should have been entirely removed from all known lipids by this hydrolysis (see Olley & Lovern, 1954, for discussion of sphingomyelin). Since we were primarily interested in the water-soluble components of the methanolic HCl phase from the countercurrent procedure, some of the $CHCl_3$-soluble material was discarded during filtration. Subsequently [see NSL (2) below] it was found that, on further hydrolysis with ethanolic KOH, this material could yield additional fatty acid. It also yielded unsaponifiable matter, which would, of course, include any sphingosine of the original lipid. In the present case, incomplete recovery of the $CHCl_3$-soluble material amounted to 10·8 g./100 g. of lipid, i.e. far in excess of that expected from the sphingosine content of the lipid (cf. Table 1). It was only analysed for N and P in OSL [Found: N 2·0, P 3·5% (atomic ratio N/P = 1·27)].

The general analytical results are given in Table 1, the distribution of P after hydrolysis is shown in Table 2 and the aldehyde determinations in Table 3. Paper chromatography of the unhydrolysed lipid in propanol–water (4:1, v/v) showed that all ninhydrin-staining material and also sugar ran with the lipid at the solvent front. After hydrolysis the sugar had the same R_F in propanol–water (4:1) as galactose, used as the control, but glucose has the same R_F in this solvent. Paper electrophoresis confirmed the identity of the glycerophosphate resulting from methanolic HCl hydrolysis and also revealed the presence of phosphorylcholine equivalent to 2·2% of the original OSL. No other organic phosphate or inorganic phosphate could be detected.

Acetylation of the unsaponifiable matter obtained by KOH hydrolysis permitted determination of the saponification equivalent (S.E.) of the non-cholesterol portion of this.

The total acetylated product had an S.E. of 358 and contained 71% of cholesteryl acetate (S.E. 428). Hence the S.E. of the non-cholesterol portion was 299, i.e. about that of the acetate of a typical long-chain aliphatic alcohol (cetyl acetate = 284). It should be noted that acetylation of this unsaponifiable matter yielded neutral acetates corresponding to only 75% of the original, the rest having become either acidic or water-soluble [see NSL (2), fraction 1, below]. The corrected value is given in Table 1.

Table 1. *Analytical data on 'old sperm lipid', as g. of component/100 g. of lipid*

	KOH hydrolysis	HCl hydrolysis	Other procedures
Fatty acids	19·6	>16·8*	—
Unsaponifiable matter (excluding aldehydes)	12·8	>8·8*	—
Cholesterol (free)	—	—	7·7
Choline†	5·5	—	10·2
Ethanolamine‡	—	0·5	—
Serine‡	—	0·07	—
Sphingosine	—	—	2·0
Reducing sugar (as galactose)	—	—	1·4
Glycerol	0·4	—	—
Inositol	—	Nil	—
N (total)	—	—	1·4
P (total)	—	—	2·15

* No recovery from $CHCl_3$-soluble fractions after countercurrent procedure (see text).

† Determined by paper chromatography after hydrolysis with KOH. Other value was determined by reineckate after baryta hydrolysis.

‡ The only additional water-soluble, ninhydrin-staining substance found by paper chromatography was glycine (0·014 g./100 g. of lipid).

Examination of 'new sperm lipid'

Only a few analyses were carried out on NSL before submitting it to chromatographic analysis. It contained 2·2% total P, 8·6% cholesterol (free) and 17·3% plasmals (modified Feulgen & Grünberg, 1938, method), and hence appeared very similar to OSL.

Chromatography of new sperm lipid (1). Chromatography was carried out on a column prepared as described by Lovern (1956*a*), except that Mallinckrodt silicic acid was used instead of silica gel (British Drug Houses Ltd.). The column, measuring 36 cm. × 4 cm., was unnecessarily large for the amount of lipid available, having been prepared before purification of the crude lipids when larger amounts had been expected. Adsorbed oxygen had been removed with 4 g. of linoleic acid (Lovern, 1956*a*). The lipid (815 mg.) was added to the column dissolved in 50 ml. of light petroleum (b.p. 40–60°), followed by 25 ml. of flask rinsings of the same solvent. Elution was effected by the following series of solvents, each being replaced when its effectiveness was seriously decreasing: light petroleum (b.p. 40–60°), ether–light petroleum (1:99; 4:96; 10:90; 25:75; 50:50; v/v, successively), ether, methanol–ether (5:95; 15:85; 25:75; 40:60; 70:30; v/v, successively), ethanol–methanol (70:30, v/v), ethanol–methanol–water (70:30:1; 70:30:5; 70:30:10, by vol., successively), ethanol–ether–water (70:30:10, by vol.). Sixty-one fractions were collected. Total recovered weight was 800 mg. The chromatographic data are illustrated in Fig. 2, where lipid concentration (mg./100 ml.) is plotted against cumulative effluent volume, and hence areas are proportional to weight of dissolved lipid.

Fractions were pooled on the basis of elution behaviour, appearance and content of aldehyde. Thus despite the apparent non-homogeneity of the material eluted between points *E* and *K*, all of it contained about the same amount of

Table 2. *Distribution of phosphorus in 'old sperm lipid' after hydrolysis*

Values are g. of phosphorus/100 g. of lipid. Total P = 2·15% (Table 1).

Hydrolysis employed	Water-sol. (total)	Water-sol. (as GPP*)	In fatty acids	In aldehydes	In unsap.	Not accounted for
KOH	0·66	0·43	0·49	Nil	Nil	1·0
Baryta	0·73	0·68	←	0·07	→	1·35
HCl	1·98†	1·45	Nil	Nil	Nil	0·17

* Glycerophosphate P was estimated by the method of Olley & Blewett (1950).

† Soluble in $CHCl_3$, 0·38 g.; soluble in water, 1·6 g. Some $CHCl_3$-soluble material was lost; 0·34 g. was attributable to phosphorylcholine (see text).

Table 3. *Aldehyde content of 'old sperm lipid' determined by various methods*

Values are g./100 g. of OSL, calculated as palmitaldehyde. Methods used are as follows: 1, modified from Feulgen & Grünberg (1938), see text; 2, Ehrlich *et al.* (1948); 3, gravimetric, see text. DMA, Dimethylacetal.

	Aldehyde in		
Method	OSL	DMA*	OSL calc. from DMA value
1	16·8, 17·3	47·0, 55·0	12·7, 14·9
2	6·9, 8·5	52·6, 57·5	14·2, 15·5
3	19·6	—	—

* Dimethylacetal-containing fraction. Yield of this fraction was 27% of OSL.

aldehyde (5–8%, colorimetrically). The final pooling and certain analytical results on the pooled fractions are given in Table 4, and the results of hydrolysis of some of them are given in Table 5. As with OSL, the distribution of phosphorus after hydrolysis is given in detail (Table 6).

Fraction 1, from its ease of elution, might be expected to consist essentially of hydrocarbons. Heptacosane has been reported as a constituent of human semen (Wagner-Jauregg, 1941). This fraction was dissolved in ether, evaporated to small volume (0·5 ml.) and treated with absolute ethanol until the solution became opalescent. On storage overnight at −10°, a white solid separated, which was centrifuged off at −10° and washed in the centrifuge with cold ethanol. Analysis showed C 84·5, H 14·1% (Weiler & Strauss, Oxford), hence it was mainly hydrocarbon. Heptacosane requires C 85·2, H 14·8%. There was insufficient material for determination of molecular weight.

Fraction 5 consisted almost entirely of free sterols. The presence of cholesterol was confirmed by isolation of the dibromide (m.p. 113–115°, authentic specimen m.p. 118°, mixed m.p. intermediate), but the yield of dibromide suggested only 70% of cholesterol in fraction 5, instead of the 89% determined colorimetrically. The optical rotation (in $CHCl_3$) of fraction 5 was $[\alpha]_D = -27{\cdot}1°$ (cholesterol $= -39{\cdot}5°$). Fast- and slow-acting sterols were estimated according to Cook, Kliman & Fieser (1954). Slow-acting sterols, i.e. cholesterol, amounted to 66%, and no fast-acting sterols were present. Since these tests suggested that considerably less cholesterol was present than the 89% originally found, the estimation was repeated, by the same colorimetric procedure, which showed 74%. A considerable period had, however, elapsed between recovery from the column (and initial colorimetric assay) and these later tests, during which time the fraction, in solution in $CHCl_3$, had been kept at −15°. That a certain amount of decomposition had occurred was evident from the fact that the stored solution, on evaporation, gave a product less completely crystalline than the original one fresh from the column.

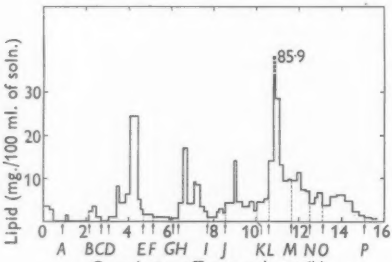

Fig. 2. Chromatography of NSL (1) on silicic acid. Solvents were changed at the points indicated by arrows. Initial solvent was light petroleum (b.p. 40–60°). Key to letters: *A–E*, ether–light petroleum (1:99; 4:96; 10:90; 25:75; 50:50 successively); *F*, ether; *G–K*, methanol–ether (5:95; 15:85; 25:75; 40:60; 70:30, successively); *L*, ethanol–methanol (70:30); *M–O*, ethanol–methanol–water (70:30:1; 70:30:5; 70:30:10 respectively); *P*, ethanol–ether–water (70:30:10). All solvent ratios are by vol.

Table 4. *Data on pooled chromatographic fractions of 'new sperm lipid' (1)'*

All analytical values are as g./100 g. of lipid. A dash indicates that no determination was made.

Fraction no.	Range (Fig. 2)	Wt. (mg.)	Appearance*	Aldehyde†	N	P	N:P (atomic)	Cholesterol
1	Origin to *A*	15·2	Colourless paste	—	—	—	—	—
2	*A–B*	1·9	White wax	—	—	—	—	—
3	*B–C*	10·4	White gum	—	—	—	—	Nil
4	*D–E* (first three fractions)	27·0	Yellow oil	—	—	—	—	Nil
5	*D–E* (next two fractions)	104·5	Crystalline at 100°	—	—	—	—	89
6	*D–E* (last two fractions)	10·6	Oil at 100°, solid at room temp.	—	—	—	—	11
7	*E–K* (except final fraction)	190·9	Brown. Pasty at 100°, wax at room temp.	6·9	0·7	1·5	1·0	—
8	*K–L* (plus fraction before *K*)	31·0	Lighter brown, phosphatidic‡	6·0	1·1	2·4	1·0	—
9	*L–M*	215·3	Lighter brown, phosphatidic‡	22·1	1·4	3·1	1·0	—
10	*M–N*	75·3	Grey powder	4·7	1·9	2·2	1·9	—
11	*N*–end	118·4	Light brown, phosphatidic‡	2·6	1·6	2·7	1·3	—
Total		800·5						

* At room temperature unless otherwise specified. † Colorimetrically on lipid. ‡ Denotes a frothy appearance at 100° and room temp., suggestive of lecithin.

Table 5. *Hydrolytic studies on certain chromatographic fractions of 'new sperm lipid* (1)'

Analytical values are as g./100 g. of lipid. A dash indicates that no determination was made.

Fraction no.	Hydrolysis employed	Fatty acids	Unsap. matter	Aldehydes*	Glycerol	Choline	Ethanolamine
3	Ethanolic KOH	22	20	—	1·1	—	—
4	Ethanolic KOH	61	33	—	0·7	—	—
7	Ethanolic KOH	32	22	9·2	0·4	Nil	1·1
9 (*a*)	Ethanolic KOH	20	11	4·6	—	8·3	—
9 (*b*)	Methanolic HCl	>14†	>10†	6·4	—	—	—
10	Aq. $Ba(OH)_2$	←———	75	———→	—	3·9	—
11	Ethanolic KOH	75	1·2	4·7	1·1	3·0	Nil

* Gravimetrically, except 9 (*b*) which was determined colorimetrically on the dimethylacetal fraction.
† See text.

Table 6. *Distribution of phosphorus after hydrolysis of certain fractions of 'new sperm lipid* (1)'

Values are as g./100 g. of original fraction.

Fraction no.	Total P	Water-sol.	Glycero-phosphate P	Inorg. P	In fatty acids	In unsap. matter	In aldehydes	Apparent loss (% of total P)
7	1·5	0·97	0·85	0·12*	0·06	Nil	Nil	32
9 (*a*)†	3·1	1·92	1·05	0·87	0·36	0·07	0·02	22
9 (*b*)†	3·1	3·07‡	1·99	0·35	Nil	←——Nil——→		1·5
10	2·2	2·00	Nil	2·00	←——Nil——→			9
11	2·7	1·56	1·28	0·28*	0·98	Nil	Nil	6

* May contain some acid-hydrolysable organic P.
† (*a*) After hydrolysis with ethanolic KOH; (*b*) after hydrolysis with methanolic HCl.
‡ Soluble in methanolic HCl (see text).

Table 7. *Chromatography of 'new sperm lipid* (2)' *on silica gel*

Fraction no.	Eluent (ml.)	Wt. (mg.)	Yield (% of original)	Appearance	Aldehyde*
1	*a*† (350)	552	67	Phosphatidic‡	18·5
2	*b* (600)	113	14	White, crystalline	2·5
3	*c* (1300)	90	11	Brown gum	2·5
4	*d* (1000)	7	1	Brown gum	—

* Direct colorimetric assay.
† *a*, Light petroleum (b.p. 40–60°); *b*, ether–light petroleum (25:75, v/v); *c* and *d*, ethanol–methanol–water (70:30:10 and 70:30:33, by vol. respectively).
‡ Denotes a frothy appearance at 100° and room temp., suggestive of lecithin.

Infrared spectroscopy confirmed that the material was mainly cholesterol, contaminated with some ketonic compound (oxidation product?).

After hydrolysis of fraction 9 with methanolic HCl, material remaining in this solvent during countercurrent distribution with light petroleum amounted to 57 g./100 g. of lipid. Of this, 26·6 g./100 g. of lipid was water-soluble and the rest $CHCl_3$-soluble. The latter material contained N 0·5 and P 2·3% (N:P = 0·48). Presumably on hydrolysis with ethanolic KOH it would have furnished additional fatty acid, and possibly also unsaponifiable matter [see NSL (2) fraction 1, below], although in the present case sphingosine is unlikely to have been present to contribute to the unsaponifiable fraction.

In view of its atomic N:P ratio of 1·9, fraction 10 was assayed for sphingosine (Found: 0·53% sphingosine N, =11·3% sphingosine).

In two cases choline was assayed both by paper chromatography and as the reineckate. The latter method gave slightly higher values (fraction 7, paper nil, reineckate 0·3%; fraction 9, paper 8·3, reineckate 8·9%). On fractions 10 and 11 only reineckate assays were made.

Chromatography of 'new sperm lipid (2)'. In view of the rather difficult elution of most of NSL (1) from Mallinckrodt silicic acid, NSL (2) was chromatographed on silica gel (British Drug Houses Ltd.). This was expected to cause the main phospholipid components to pass through without fractionation, but to retain cholesterol, waxes, triglycerides, etc. (Lovern, 1956*a*). The column contained 30 g. of mixed silica gel and Hyflo Super-cel prepared as described by Lovern (1956*a*). NSL (2) (826 mg.) was added to the column in 50 ml. of light petroleum. The elution data are given in Table 7. No further material could be removed from the column by a mixture of equal parts of ethanol, methanol and water.

Fraction 1 contained 18·5% aldehydes, fractions 2 and 3 each 2·5% aldehydes by colorimetric assay before hydrolysis. Other analytical data are presented in Table 8. Reference has been made earlier to the fact that on hydrolysis with methanolic HCl, the material passing into the

Table 8. *Analytical data* on fractions of 'new sperm lipid* (2)'

Values are as g./100 g. of lipid. A dash indicates that no determination was made.

Fraction no.	Fatty acids	Unsap. matter	Aldehydes	Cholesterol	Choline	Total P	Glycero-phosphate P	Ethanol-amine
1	28·9, 29·7‡	21·6†, 19·5‡	20·0, 18·6	—	6·5	3·1	1·1	0·07
2	34·5	11·4	—	43·6	—	—	—	—
3	25·7	27·1	5·7	6·3	4·6	1·7	1·4	—

* Fraction 1 was hydrolysed with both ethanolic KOH and methanolic HCl (first and second values respectively for fatty acids, unsaponifiable matter and aldehydes). Fractions 2 and 3 were hydrolysed only with ethanolic KOH. Aldehydes were determined gravimetrically after hydrolysis with KOH, colorimetrically on dimethylacetals in total unsaponifiable material after hydrolysis with HCl. Unsaponifiable material was exclusive of aldehydes (1 and 3) or cholesterol (2 and 3). Glycerophosphate and choline were determined in fraction 1 after KOH hydrolysis. Choline was determined by reineckate.

† May not be fully hydrolysed; see text. 'Corrected' value = 10·0.

‡ Corrected for material obtained on subsequent hydrolysis with KOH; see text.

methanol phase on subsequent partition between light petroleum and methanolic HCl is not entirely soluble in water. The water-insoluble material is quite insoluble in light petroleum, but readily soluble in $CHCl_3$. In fraction 1 above, a total methanol-phase product amounting to 42·7% of the original lipid was obtained, of which very little (5·9% of the original lipid) was water-soluble. Hydrolysis of the $CHCl_3$-soluble material with ethanolic KOH yielded 23·4% of fatty acids and 16·9% of unsaponifiable material, the rest becoming water-soluble. Calculated back to the original lipid of fraction 1, these additional amounts of fatty acid and unsaponifiable matter amount to 6·1 and 4·4% respectively. This addition has been made in the data given in Table 8 and, as will be seen, brings the results of KOH and HCl hydrolysis respectively into close agreement.

On acetylation of the unsaponifiable matter obtained by KOH hydrolysis of fraction 1 there was obtained neutral material 10, acidic material 3·6 and water-soluble material (by difference) 8 g./100 g. of lipid. This should be compared with the similar, but smaller, production of water-soluble material on refluxing the unsaponifiable matter similarly obtained from OSL with acetic anhydride. The presence of much cholesterol in the latter material and its absence in the former could account for the quantitative difference.

RESULTS

Three sets of data are available for evaluation: on an unfractionated product (OSL), on a product only slightly fractionated by chromatography (NSL, 2) and on a large range of chromatographic fractions (NSL, 1). There is evidence, from obvious discrepancies in aldehyde values before and after chromatography, of degradative changes occurring during the chromatography of NSL (1). Detailed discussion of these and of other discrepancies or unexpected analytical findings is deferred to the following section of the paper. For present purposes the discrepancies have been accommodated by making certain assumptions.

Since aldehydogenic compounds appear to be major components of the sperm lipids it may be desirable at the outset to confirm that the relevant analytical data do, in fact, relate solely to higher aliphatic aldehydes, as found in plasmalogens (acetal phosphatides). Spermatozoa are rich in deoxypentose, which will react with the Feulgen reagent for aldehydes. It is unlikely that appreciable proportions of such material would remain after the purification procedure (Fig. 1), but in any case the coloured product produced from deoxyribose is not extracted from the aqueous phase into $CHCl_3$. This latter behaviour seems to be typical of aldehydes with lipid-like solubility properties, the lower aliphatic aldehydes, e.g. formaldehyde, behaving like deoxyribose. The aldehyde was not only determined colorimetrically; the total amount could be recovered as dimethylacetals, again with the solubility properties of a lipid. Finally, the free aldehyde could be recovered quantitatively by a process which implies an original linkage into a lipid of the plasmalogen type, i.e. capable of forming a water-soluble alkali plasmalogenate. In connexion with this last method of assay it may be objected that the product shows a variable or even no reactivity towards the Feulgen reagent (cf. Olley & Lovern, 1953; Lovern & Olley, 1953*b*). This loss of activity can, in the present instance, be attributed to polymerization, since the material could be depolymerized and rendered active to the Feulgen reagent by distillation in a molecular still. Extensive polymerization of free palmitaldehyde has been found to occur during manipulations similar to those employed in the gravimetric assay.

For purposes of calculation it is accepted that plasmalogens contain one fatty acid radical and one aldehydogenic radical attached to a glycerophosphate–base unit (Lovern, 1956*b*; Debuch, 1956). For a choline-based plasmalogen of Debuch's formula, assuming fatty acids and aldehydes of 18 and 16 carbon atoms respectively, the yields of hydrolytic products would be: fatty acids 36·3, aldehydes 30·5, choline 15·45 and glycerophosphate P 3·96 g./100 g. of lipid. The alternative structure of Rapport, Lerner, Alonzo & Franzl (1957) would give virtually the same yields.

Composition of 'old sperm lipid'

Certain hydrolytic products were present in such small proportions (Table 1) that quantitative assessment of their significance is inadvisable. Such are, for example, glycerol and sphingosine. Chromatography of NSL (1), see below, suggests that not all the glycerol is derived from triglycerides. That the spingosine probably represents sphingomyelin rather than cerebrosides is suggested by the finding of phosphorylcholine in the HCl hydrolysate, and by chromatography of NSL (1). At 2%, the sphingosine is equivalent to 0·21% phosphorylcholine (found 0·34%) and to 5·5% sphingomyelin. On this assumption the reducing sugar must be ignored as a possible lipid component. The total non-aldehydic unsaponifiable matter, after deduction of cholesterol, amounts to 5·1%. This will include hydrocarbons [about 2 g./100 g. of lipid from chromatographic data on NSL (1) below] and possibly ceramides (fatty acid amides of sphingosine) after KOH hydrolysis. Part of the sphingomyelin will, however, pass into the fatty acid fraction (Lovern & Olley, 1953*b*). Ceramides would yield a diacetyl derivative on acetylation (S.E. of diacetyl-lignocerylsphingosine = 347, the amide link being resistant to KOH). Therefore, although the S.E. of the (acetylated) non-cholesterol portion of the unsaponifiable matter (299) approximates to that of a typical long-chain aliphatic alcohol (cetyl acetate = 284), the presence or likely proportion of such compounds cannot be inferred from it.

If the fatty acids (19·6%, Table 1) are present entirely as plasmalogens, they are equivalent to 16·5% aldehydes. Neglecting the inexplicably low value by method 2 on the original lipid (but not on the dimethylacetal fraction), and assuming that recovery of dimethylacetals is not absolutely quantitative, the data in Table 3 suggest that, in fact, aldehyde is present equivalent to the total fatty acids. Choline is the only important base. Taking the higher value for choline (see Discussion), the total bases found account for 1·45% N, i.e. the total present. If all the fatty acid is present as plasmalogen, the base requirement is equivalent to 8·3 g. of choline/100 g. of lipid, i.e. to most of that present. On the same assumption, the glycerophosphate requirement is equivalent to 2·1% P. Although there is sufficient total P (2·15%), there is a marked lack of glycerophosphate P (Table 2). Leaving consideration of this discrepancy for later discussion, it may be said that the data on OSL suggest that the chief component is a choline-based plasmalogen, the amount being about 54 g./100 g. of lipid. Allowing for the other postulated lipids, there appears to be about 25% of non-lipids present. This may be impurity, or may represent some unknown component of the plasmalogen (see Discussion).

Composition of 'new sperm lipid (2)'

It seems preferable to consider the chromatography of NSL (2) before that of NSL (1) since it forms a closer link with the unfractionated OSL. In attemping to evaluate the composition of chromatographic fractions, whether of NSL (1) or NSL (2), account has been taken not only of the analytical data but of previous findings of the order of elution of various lipids from silicic acid or silica gel (Borgström, 1952*a*, *b*; Rapport, Lerner & Alonzo, 1953; Fillerup & Mead, 1953; Mead & Fillerup, 1954; Lea, Rhodes & Stoll, 1955; Spengler & Hauf, 1955; Lovern, 1956*a*). The fractions are considered individually below.

Fraction 1. Eluent, light petroleum; yield 67%. The major analytical values (Table 8) again suggest that a choline plasmalogen is the main component. The aldehyde (19%) is equivalent to 22·6 g. of fatty acid/100 g. of lipid. This leaves an excess of only about 6–7 g./100 g. available for other lipids. Choline, calculated from the aldehyde value, should be 9·6 g./100 g., i.e. in excess of that liberated by KOH. Unfortunately lack of material precluded additional hydrolyses with baryta on chromatographic fractions. Glycerophosphate P should be 2·5 g./100 g., i.e. about twice that liberated by KOH and approaching the total P of the fraction, as with OSL. The content of plasmalogen, calculated from the aldehyde content, is about 62%. An interesting feature is the presence of a considerable amount of non-aldehydic, non-sterol 'unsaponifiable' matter. The incomplete splitting of this material by ethanolic KOH, as indicated by the results of acetylation, has been described. Evidently methanolic HCl is no more effective in this case. There was insufficient acetylated product for determination of S.E. but, in any case, the same reservations would apply as in the case of OSL. Allowing for the additional 3·6% of (presumed) fatty acids obtained after acetylation of the crude 'unsaponifiable' matter, the fatty acids in excess of plasmalogen requirements amount to 10 g./100 g. of fraction, i.e. the same as the 'corrected' value for unsaponifiable matter in Table 8. This ratio is suggestive of wax esters but, in view of the ready elution from silica gel, the nature of the non-plasmalogen lipid components is obscure. There appears to be about 20% of non-lipid material (impurity?) in the fraction, similar to the position with OSL.

Fraction 2. Eluent, ether–light petroleum (1:3, v/v); yield 14%. This fraction contains, as would be expected, almost the total cholesterol (6·1 g./100 g.) of NSL (2). (By accident, elution was not carried to completion before changing the solvent

for collection of fraction 3.) The fatty acids may be present partly as wax esters (say 22 % of such esters), but part (about 23 g./100 g. of fraction) must be present in some other combination, or as free acids (see final paragraph of Discussion, page 642). Non-lipids not more than 10 %.

Fraction 3. Eluent, ethanol–methanol–water (70:30:10, by vol.); yield 11 %. The main components might appear to be waxes, were it not for the strong adsorption. There are also small amounts of aldehydogenic lipids, which could be choline-based. However, since the main bulk of these behaves like lecithin in not being adsorbed by silica gel, the material in fraction 3 must be quite different from that in fraction 1. In fact, it recalls the aldehydogenic material found in NSL (1) fraction 11 (see below). Non-lipids may be more important than in fractions 1 and 2, but cannot be estimated apart from an upper limit of about 30 %.

Composition of 'new sperm lipid (1)'

Fraction 1. Eluent, light petroleum; yield 1·9 %. This consisted of hydrocarbons.

Fraction 2. Eluent, ether–light petroleum (1:99, v/v); yield 0·2 %. This fraction was not examined.

Fraction 3. Eluent, ether–light petroleum (4:96, v/v); yield 1·3 %. The analytical data suggest a wax ester, in agreement with the elution behaviour. The glycerol could be from triglycerides (11 % of the fraction), leaving 12 % fatty acids and 20 % unsaponifiable matter. The ratio of these last two components suggests the presence of about 8 % free alcohols and 24 % wax esters, leaving 57 % of the fraction as non-lipids.

Fraction 4. Eluent, ether–light petroleum (25:75, v/v); yield 3·4 %. The glycerol may represent triglycerides (7 %). The unsaponifiable matter (higher alcohols?) could represent about 66 % wax esters, leaving about 22 % excess of fatty acids, of unknown origin, but possibly free (see final paragraph of Discussion, page 642). Non-lipids not more than 5 %.

Fraction 5. Eluent, as for fraction 4; yield 13 %. Free cholesterol, 89 %. Other components were not examined.

Fraction 6. Eluent, as for fraction 4; yield 1·3 %. Free cholesterol, 11 %. Other components were not examined.

Fraction 7. Eluent, a range of solvents from ether–light petroleum (50:50, v/v), through ether, up to methanol–ether (40:60, v/v); yield 23·9 %. The aldehyde may represent an ethanolamine-based plasmalogen (elution behaviour). In this case it would require 1·8 % ethanolamine (found 1·1 %), 0·90 % glycerophosphate P (found 0·85 %) and 8·2 % fatty acids (found 32 %), taking the direct colorimetric value for aldehyde. Accepting that it is an ethanolamine-based plasmalogen, the amount is about 20 % of the fraction. The unsaponifiable matter might again represent a wax ester (44 % of the fraction) leaving just under 2 % of fatty acids for other lipids. These could be mono- or di-glycerides (triglycerides should have been eluted). On this basis non-lipids are about 34 %. The appearance of wax esters so late in the elution series is surprising (Spengler & Hauf, 1955), but the presence of much non-lipid, possibly combined in some way with the lipids, may markedly affect their chromatographic behaviour. Alternatively, it is necessary to postulate the presence of a novel, non-phosphatidic lipid or lipids.

Fraction 8. Eluent, initially methanol–ether (40:60, v/v) but mainly methanol–ether (70:30, v/v); yield 3·9 %. No hydrolysis was performed, hence little calculation is possible. The fraction was richer in N and P than fraction 7, possibly containing a little phospholipid additional to plasmalogens (about 18 %, from aldehyde content).

Fraction 9. Eluent, ethanol–methanol (70:30, v/v); yield 27 %. The probable presence of choline-based plasmalogens has been discussed above, the amount, based on the aldehyde content, being about 72 % of the fraction. There is a small deficiency of fatty acids (see final paragraph of Discussion, page 642), which would be equivalent to 55 % plasmalogens. The choline would likewise be equivalent to 54 % plasmalogens but liberation by KOH is probably incomplete (see Discussion). The fatty acids are equivalent to 16·8 % aldehydes. Glycerophosphate P equivalent to the aldehyde content would be 2·9 g./100 g. of lipid (see Discussion). If the unsaponifiable matter obtained on KOH hydrolysis (11 %) is really polymerized aldehydes, the total aldehyde content by gravimetric assay would be about 15·6 %. There may well therefore be no non-aldehydic unsaponifiable matter, and there is certainly no other evidence of any lipid additional to a choline-based plasmalogen. There must, however, be 30–40 % of non-lipid material.

Fraction 10. Eluent, ethanol–methanol–water (70:30:1, by vol.); yield 9·4 %. This fraction appears to contain sphingomyelin. It does not yield any glycerophosphate on hydrolysis, and its aldehyde component cannot, on present knowledge, be equated with the other components. The choline is equivalent to 91 % of the sphingosine found and to 1·0 % P (sphingosine equivalent to 1·2 % P). The sphingomyelin content is about 30 %. The rest of the fraction appears to be lipid in nature, since the $CHCl_3$ extract after hydrolysis (McKibbin & Taylor, 1949) amounted to 75 g./100 g. of fraction, about the same as for sphingomyelin itself. This remaining lipid may also have an N:P ratio of 2 (calc. 1·85), but its nitrogen becomes water-soluble after hydrolysis. Its phosphorus, like that of

sphingomyelin, is liberated as orthophosphate on hydrolysis. However, this remaining 70% of fraction 10 is low in both N and P (0·9 and 1·1% respectively).

Fraction 11. Eluent, ethanol–methanol–water (70:30:5 and 70:30:10, by vol.) and ethanol–ether–water (70:30:10, by vol.); yield 14·8%. The composition of this fraction cannot be elucidated. It could contain some choline-based plasmalogen, but it is not clear why such should have resisted earlier elution. The re-appearance of glycerophosphate as a component, after a complete break in fraction 10, suggests a particularly strongly adsorbed type of phospholipid, such as lysolecithin. Loss of aldehyde by hydrolysis on the column, as discussed below, could yield lysolecithin. However, there is far too much fatty acid to balance the available choline and glycerophosphate, and some lipid other than a lysophospholipid must predominate. The glycerol obtained on KOH hydrolysis is also difficult to explain. Monoglycerides, although more strongly adsorbed than triglycerides, would hardly be expected so late in the sequence.

DISCUSSION

Although the presence of a choline-based plasmalogen as the major component of the lipids of ram spermatozoa is suggested by the foregoing results, various discrepancies and unexpected analytical findings must be indicated. These are discussed under appropriate subheadings before considering other aspects of the overall composition of these lipids.

Chromatographic losses

Although reasonably complete recovery was achieved of the total weight of material put on the column, extensive loss of the postulated plasmalogen occurred during chromatography of NSL (1). This operation involved a total elution time of 3 weeks, fraction 9 having been on the column a little over 2 weeks. It may be noted that Skipski & Rapport (1957) find it necessary to wash silicic acid-impregnated paper with pyridine before chromatography of plasmalogens, to avoid liberation of the aldehyde. Fraction 9, which, from both the nature of the eluting solvent and from its analytical characteristics, corresponds to the main choline–plasmalogen peak, amounted to only 27% of the original lipids, and its aldehyde content (22·1%, Table 4) was little higher than that of the starting material (17·3%). A sharp separation should have yielded a fraction amounting to 57% of the total, and containing about 30% aldehyde. Although minor concentrations of aldehydogenic lipid are present in other fractions, the total aldehyde content of NSL calculated from the data in Table 4 is 8·5 g./100 g. of lipid, i.e. about half the original. This cannot be explained by non-elution from the column.

A further point to be noted is that, although the aldehyde content of fraction 9 determined colorimetrically was 21%, after hydrolysis only 4·6% could be recovered gravimetrically or 6·4% as dimethylacetals (Table 5). Other fractions of NSL (1), by contrast, gave gravimetric recoveries of the same order as, and indeed somewhat higher than, those obtained by direct colorimetric assay. An interval of some 3 weeks had elapsed between elution from the column, with prompt colorimetric determination of aldehyde, and the hydrolyses, during which time the fractions had been kept in solution in chloroform at 0°. It is difficult to see why decomposition should have affected only fraction 9, and it is difficult to envisage any decomposition other than rupture of the acetal linkage which could interfere with the subsequent determination of the aldehyde. If, however, this link were broken, the free aldehyde would be estimated as non-aldehydic unsaponifiable matter by the gravimetric method and polymerization could account for its incomplete recovery as the dimethylacetals. Other analytical characteristics of the fraction should be unaltered. G. V. Marinetti (personal communication) has found that plasmalogens are unstable in chloroform solution.

If loss of aldehyde on the column is also due to cleavage of the acetal linkage during the very lengthy adsorption on an acidic medium, followed by polymerization of the free aldehyde, the total fatty acids recovered from the column should not show a comparable fall, the apparent non-aldehydic unsaponifiable matter should show a rise, and lysolecithin should be encountered. The total fatty acids of fractions 3, 4, 7, 9 and 11, the only ones for which figures are available, amount to 26·4 g./100 g. of original lipid, the true total being, of course, slightly higher; the fatty acid content of NSL calculated from Table 8 is 29·4 g./100 g. It is unfortunately impossible to test in the same way whether there has been a rise in unsaponifiable matter. The difficulty is that, as mentioned above, hydrolysis with ethanolic potassium hydroxide gave an 'unsaponifiable' fraction which, on acetylation, yielded a considerable proportion of water-soluble material. Only a few fractions were acetylated, so that some of the data for unsaponifiable matter may be seriously in error. Lysolecithin may have occurred in fraction 11, but in nothing like an amount equivalent to the aldehyde 'lost' during chromatography. For example, the glycerophosphate of fraction 11 is equivalent to about only one-sixth of this aldehyde.

However, as will be discussed below, the constitution of the sperm-lipid plasmalogen is uncertain and it may not give a simple lysophospholipid on

loss of the aldehyde. A significant finding may be the high fatty acid content (75%, Table 5) of the unidentified lipid of fraction 11. This fatty acid amounts to about 11 g./100 g. of original lipid, i.e. it is roughly equivalent to the missing aldehyde and the lipid in which it is bound may be the residual portion of the original plasmalogen.

Chromatography of NSL (2) did not result in any serious loss of aldehyde, recovery being equivalent to about 15 g./100 g. of lipid, compared with the initial value of 17·3 g./100 g. In this case, however, elution was rapid (total elution time 2 days; fraction 1 about 1 hr.). There was also no loss of aldehyde between recovery from the column and subsequent hydrolysis, which followed within 24 hr. in this case. It may be noted that fraction 3 of NSL (2), which corresponds to fraction 11 of NSL (1), although showing similarity in its content of strongly adsorbed aldehyde, choline, glycerophosphate, etc. (Table 8), did not contain the high proportion of fatty acids which, it has been suggested above, may be present in the residue of a degraded plasmalogen.

Behaviour on hydrolysis

Although there does not seem to be any published record of hydrolysis studies on a pure natural plasmalogen, a compound of Debuch's (1956) formula would be expected to yield all its choline as readily to ethanolic potassium hydroxide as does lecithin. Furthermore, removal of base is postulated during the gravimetric aldehyde assay, which was quantitative. Although, unfortunately, lack of material prevented studies of baryta hydrolysis on the various fractions, experience with OSL showed that potassium hydroxide liberated only about half as much free choline as did baryta (Table 1). The difference cannot be attributed to sphingomyelin, for which it is known that aqueous baryta is a more effective hydrolytic agent than is ethanolic potassium hydroxide. The amount of choline found after baryta hydrolysis is in agreement with the total nitrogen of the lipid and is approximately equivalent to the aldehyde (if this is present as a choline plasmalogen).

In all cases sufficient total phosphorus is present to supply one phosphorus atom/molecule of aldehyde. However, the amount found as glycerophosphate after hydrolysis with potassium hydroxide, followed by acidification, is far below this requirement [see particularly the results on OSL, NSL (1) fraction 9 and NSL (2) fraction 1]. Baryta hydrolysis is little more effective in this respect (Table 2). Methanolic hydrochloric acid liberates considerably more glycerophosphate, although still not enough to balance the aldehyde. Some decomposition of glycerophosphate may be expected with methanolic hydrochloric acid, e.g. Olley & Lovern (1954) found 35% hydrolysis of glycerophosphate during refluxing for 8 hr. with methanolic 6N-hydrochloric acid. Allowing for 26% hydrolysis during the refluxing for 6 hr. in the present work, the total recovery of glycerophosphate after hydrolysis of OSL with hydrochloric acid (Table 2) is 1·96 g./100 g. and for NSL (1) fraction 9 (Table 6) is 2·6 g./100 g. [It must be noted, however, that the expected inorganic phosphate was not detected (by paper electrophoresis) with OSL, and less than the calculated amount was found, chemically, with NSL (1) fraction 9.] These values agree fairly well with the aldehyde requirements for glycerophosphate phosphorus (2·1 and 2·9 g./100 g. respectively). No determination was made of glycerophosphate liberated by hydrochloric acid from NSL (2) fraction 1.

Certain other features observed on hydrolysis should be noted. Hydrolysis with ethanolic potassium hydroxide was incomplete in respect of items additional to choline and glycerophosphate. A large yield of 'unsaponifiable' matter was obtained, part at least of which could be further degraded by refluxing with acetic anhydride, when a considerable proportion became water-soluble [see particularly NSL (2) fraction 1]. Acetic anhydride is known to be an effective dephosphorylating agent for phospholipids (Bevan, Brown, Gregory & Malkin, 1953) and this present effect indicates that it can also attack other types of linkage. The fatty acids recovered after hydrolysis with potassium hydroxide contained appreciable amounts of some phosphorus compound, e.g. from OSL and NSL (1) fractions 9 and 11 the fatty acids contained 2·5, 1·8 and 1·3 g. of phosphorus/100 g. of acids respectively (Tables 2 and 6). These values are far higher than those commonly found after alkaline hydrolysis of glycerophospholipids (e.g. Trier, 1913; Olley & Lovern, 1953); a common value, in our experience, is about 0·1–0·2% phosphorus. It is improbable that sphingomyelin is present in sufficient amount in these fractions to account for this acidic lipid phosphorus. The absence of phosphorus from fatty acids recovered after hydrolysis with methanolic hydrochloric acid may be noted. G. V. Marinetti (personal communication) has also found that phosphorus-containing acidic lipids accompany the fatty acids obtained after alkaline hydrolysis of plasmalogens. The distribution of phosphorus after hydrolysis is discussed further below.

Countercurrent distribution of the products of such hydrolysis with methanolic hydrochloric acid, however, yielded a product from the methanolic phase which could be further hydrolysed with ethanolic potassium hydroxide [see especially NSL (2) fraction 1]. This material could not have been simply a portion of the original lipid which

had escaped hydrolysis, since it was insoluble in light petroleum. Recovery of dimethylacetals was sufficiently quantitative, however, to confirm that liberation of aldehyde by methanolic hydrochloric acid was complete.

There is, of course, no direct evidence that any or all of these aspects of incomplete hydrolysis relate to the postulated plasmalogen rather than to possible accompanying substances. Nevertheless, if the plasmalogen accounts for most of the fatty acid, choline and glycerophosphate of the total lipids, the above findings suggest that its structure may be unusual. It resembles the usual plasmalogens in one important respect: its reaction with the fuchsin reagent was rapid at room temperature, in contrast with the slow reaction of the dimethylacetals (cf. Lovern, 1956*b*).

The presence of large proportions of water-soluble products of hydrolysis other than known lipid fragments such as choline, glycerophosphate, etc., may be noted. Provisionally called 'non-lipid', these substances amounted to about 25, 20 and 35% of OSL, NSL (2) fraction 1 and NSL (1) fraction 9 respectively. This material accompanied the lipid through the various purifications employed (Fig. 1) and the main plasmalogen peak during chromatography on silicic acid. Part of it was still linked in a lipid-like substance after hydrolysis with ethanolic potassium hydroxide (the crude 'unsaponifiable' fraction) or hydrolysis with methanolic hydrochloric acid (the chloroform-soluble portion from the methanolic hydrochloric acid phase after countercurrent distribution). This may, perhaps, be another indication that the sperm plasmalogen is a more complex molecule than the usual type.

Distribution of phosphorus after hydrolysis

Two peculiarities in respect of phosphorus have already been noted, namely the occurrence of phosphorus in acidic lipid form and the low yield of glycerophosphate, both after hydrolysis with potassium hydroxide. The latter finding cannot be attributed entirely to the former, since even adding the phosphorus of the fatty acid fraction to that found as glycerophosphate, the total such phosphorus after hydrolysis with potassium hydroxide is considerably less than that found as glycerophosphate after acid hydrolysis (Tables 2 and 6). After acid hydrolysis, a considerable amount of phosphorus remains in the chloroform-soluble water-insoluble fraction. A notable feature of alkaline hydrolysis, whether with ethanolic potassium hydroxide or with aqueous baryta, is that much of the phosphorus is analytically unaccounted for after hydrolysis. Such 'loss' is very small after acid hydrolysis.

In one case [NSL (2) fraction 1], total phosphorus was determined immediately after hydrolysis with potassium hydroxide, before working up the hydrolysate. The value obtained was identical with that on the intact lipid. A typical plasmalogen should be present as potassium plasmalogenate at this stage. Subsequent acidification during the working up of the hydrolysate would liberate glycerophosphate from the usual type of plasmalogenate, but we know that in the present case glycerophosphate production after hydrolysis with potassium hydroxide is incomplete. It is suggested that the balance of the lipid phosphorus is liberated from the sperm 'plasmalogenate' as a compound from which it is not converted into orthophosphate by the digestion procedure used.

Failure to recover all the original phosphorus of a lipid among the products of hydrolysis has been reported before (Olley & Lovern, 1953) and has been attributed to difficulty in digesting some forms of organic phosphorus (Olley, 1956*a*). The difficulty does not arise with, for example, lecithin, and glycerophosphate offers no digestion problem. It was believed from this earlier work that the digestion procedure used in the present case (basically the substitution of perchloric acid for sulphuric acid in the method of Umbreit, Burris & Stauffer, 1949) was adequate. Again glycerophosphate offers no difficulty. In compiling the data in Tables 2 and 6 no material, e.g. water washings, was discarded without analysis and satisfactory replication was invariably obtained in all determinations of phosphorus. These findings further emphasize the apparently novel character of some major component of the sperm lipids, i.e. of the postulated plasmalogen.

General composition of sperm lipids

Apart from a choline-based plasmalogen, the sperm lipids contain cholesterol (about 8%), apparently all free, sphingomyelin [about 5·5%, based on the sphingosine content of OSL, about half of this total occurring in fraction 10 of NSL (1)], hydrocarbon (about 2%) and small amounts of lipids based on ethanolamine (possibly also a plasmalogen) and glycerol. There may be some aliphatic alcohols (or wax esters) and perhaps a little glycolipid. If the plasmalogen resembles the usual type, the amount appears to be about 55–60 g./100 g. of 'purified' lipids, the balance of the total material then representing impurities (see above). There is no evidence for the occurrence of lecithin in these lipids. The absence of inositol lipids requires confirmation, since hydrolysis of washed, freeze-dried ram spermatozoa with aqueous 6N-hydrochloric acid showed the presence of 0·11% inositol. It is possible that inositol lipids were lost by the washing process (Folch *et al.* 1951) or by adsorption on the cellulose column (cf.

Garcia, Lovern & Olley, 1956). Alternatively, the bound inositol may have been insoluble either because it was of non-lipid nature or because it was present as a lipoprotein insoluble in chloroform–methanol (Folch, 1952; Hutchison *et al.* 1956).

It has been found (E. F. Hartree and T. Mann, unpublished experiments) that ram spermatozoa contain a phospholipase *A* able to liberate the fatty acid from endogenous plasmalogen (cf. Franzl & Rapport, 1955). This enzyme would have been active during the interval between semen collection and freeze-drying of the washed sperm. It is possible to make a rough assessment of the upper limit of such enzymic hydrolysis from the amount of free fatty acids detected chromatographically. Small amounts of such acids probably occurred in NSL (1), fraction 4, but following fractions, not fully characterized, may have contained additional amounts. In the chromatography of NSL (2) any free fatty acids would be quantitatively concentrated in fraction 2. If the entire fatty acid found after hydrolysis of this fraction had been free in the intact fraction, it would amount to 4·8% of the original lipid, representing hydrolysis of the plasmalogen component to the extent of about 23%. This is therefore the upper limit and a more likely value, allowing for possible wax esters in NSL (2), fraction 2, would be about 15% hydrolysis. The deficiency of fatty acids relative to aldehydes in the most selectively fractionated plasmalogen preparation, NSL (1), fraction 9, amounts to 6·3 g./100 g. of fraction. Assuming that the chromatographic behaviour of enzymically degraded and undegraded plasmalogen is the same, and ignoring the extensive chromatographic losses, this fatty acid deficiency corresponds to about 24% hydrolysis of the original plasmalogen. It is unlikely that hydrolysis to the extent of about 20% would have seriously affected the overall assessment of the composition of the sperm lipids, but it may have contributed to some of the unexpected experimental observations, since the properties of the lysoplasmalogen have not been studied in any detail.

SUMMARY

1. Washed freeze-dried ram spermatozoa were extracted with chloroform–methanol (2:1, v/v) and the crude extracts purified by washing with water and passing through a column of cellulose, the yield of total lipids being 7–9%.

2. Part of this material was hydrolysed directly, ethanolic potassium hydroxide, methanolic hydrochloric acid and aqueous barium hydroxide being used as alternative reagents, and part was examined chromatographically with silicic acid (or silica gel) from two sources, these being products known to have widely different adsorptive properties.

3. From analytical data on the original lipids and on the various chromatographic fractions it is evident that aldehydogenic lipids, possibly plasmalogens, are major components (about 55–60%) of the sperm lipids. Choline is the predominating base of these particular compounds, although there are minor amounts of ethanolamine. The behaviour towards hydrolytic agents is, however, not that to be expected from the accepted plasmalogens.

4. There is no evidence for the presence of lecithin. Other components of the extracts are free cholesterol (about 8%), hydrocarbons (about 2%), probably sphingomyelin (about 5·5%) and other lipids difficult to characterize, but perhaps including wax esters and glycerides. Despite the purifications employed, non-lipids appeared to be present in appreciable amount, possibly combined with the lipids.

5. Both ethanolic potassium hydroxide and methanolic hydrochloric acid proved inadequate for the complete hydrolysis of some of the lipids. The former sometimes gave 'fatty acids' rich in phosphorus and 'unsaponifiable matter' which became largely water-soluble on refluxing with acetic anhydride. The latter gave a fraction susceptible to further hydrolysis with ethanolic potassium hydroxide.

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Hydrolysis of the Soluble Pentosans of Wheat Flour and *Rhodymenia palmata* by Ruminal Micro-organisms

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It has been known for some time that pentosans in the food are to a large extent decomposed during passage through the rumen of sheep (Marshall, 1949; Heald, 1953). This microbial activity enables the ruminant to make use of pentosans and other hemicelluloses which, like cellulose, are hardly, if at all, available to non-ruminants. Heald (1952) also isolated some xylose-fermenting strains of *Escherichia coli* from the rumen but, when the work about to be described was undertaken, nothing was known of the bacteria in the rumen responsible for fermenting the pentosans themselves. Two species of pentosan-fermenting bacteria have recently been found in the rumen; both appear to be hitherto undescribed species of nutritionally exacting, obligately anaerobic, Gram-negative, curved rods (Bryant & Small, 1956; Doetsch, Howard, Mann & Oxford, 1957).

To assist in an understanding of the processes of pentosan digestion in the sheep rumen, experiments were undertaken *in vitro* with suspensions of the natural mixed culture of bacteria found in the rumen of a sheep which was maintained on a standard diet containing a fair amount of pentosan. It was considered that pentosans which were water-soluble and could be obtained fairly readily in a state of purity would present the most convenient type of substrate for preliminary studies. Two such materials have been examined so far, the soluble pentosan of wheat flour and the soluble xylan of the red seaweed *Rhodymenia palmata*. The former was first isolated by Perlin (1951*a*) from commercial

wheat flour. Perlin (1951*b*) showed the polysaccharide to have a main chain of D-xylopyranose units, linked β-1:4, with single L-arabofuranoside units attached along the chain at positions 2 and 3 of the xylose. About 300 anhydropentose units were present in each molecule. In the present work, material very similar in properties to Perlin's pentosan has been obtained by cold-water extraction of unbleached untreated wheat flour. In preparing large amounts of hexosan-free material for these studies a new method of purification has been developed, based on the observation that the hexosan present in the crude material is susceptible to the hydrolytic action of saliva whereas the pentosan component is not. This enzymic purification of the pentosan affords proof, additional to Perlin's (1951*a*) chemical separation, that the pentose and hexose in the water-soluble polysaccharides of wheat flour exist in separate pentosans and hexosans, and not in mixed polysaccharides.

The xylan of *Rhodymenia palmata* was first isolated by Barry & Dillon (1940), and subsequent chemical investigations have been reported by Percival & Chanda (1950), Barry, Dillon, Hawkins & O'Colla (1950) and Hirst (1955). The polysaccharide molecule is relatively small, consisting of only 16 or 17 xylopyranose units. The unique feature of this xylan is that although most of the xylose units are linked in β-1:4 fashion as in xylans generally, about one linkage in five is of the β-1:3 type. Nothing appears to be known about the distribution of these linkages along the chain.

The present communication is concerned with the hydrolytic activities of toluene-treated suspensions of mixed rumen bacteria, particular attention being paid to the oligosaccharide intermediates in the hydrolysis of the two substrates. Some hitherto unknown xylose oligosaccharides have been obtained from the *Rhodymenia* xylan, and their chemical constitutions have been investigated. It is proposed that the name *rhodymenan* be given to this unique polysaccharide, and that the characteristic disaccharide obtained from it, containing two xylose residues linked β-1:3, and which is described below, be known as *rhodymenabiose*. The contribution which this investigation makes to knowledge of the structure of rhodymenan is considered. The only xylose oligosaccharides known hitherto have all belonged to the β-1:4 series (Whistler & Tu, 1952), and the simple names xylobiose, xylotriose, etc., have already been appropriated for these substances. That part of the present work dealing with the wheat-flour pentosan has already been briefly reported (Howard, 1955).

METHODS AND MATERIALS

Sheep used as a source of rumen bacteria. The sheep used throughout this investigation was a Cheviot wether, no. 879, fitted with a rumen cannula, and fed daily with a mixture of hay (750 g.) and concentrates (450 g., containing chiefly ground maize, crushed oats and bran). This was the sheep from which Doetsch *et al.* (1957) isolated the pentosan-fermenting organism *Bacteriodes amylogenes*.

Preparation of suspensions of rumen bacteria. Quantities of approximately 1 l. of rumen liquor were removed through the cannula about 2 hr. after feeding, and mixed with sodium sulphide solution to give a final concentration of 0·02% $Na_2S,9H_2O$ in order to maintain anaerobic conditions during manipulation. Larger food particles were removed by straining through several layers of surgical gauze; Protozoa and small food particles were removed by centrifuging at 300 *g* for 1 min. The supernatant solution, containing large numbers of different eubacteria, but only very few of the smaller Protozoa and larger bacteria, was spun at 4500 *g* for 30 min. The almost clear supernatant was discarded, and the cells were resuspended in the buffer solution described by Elsden (1946), ammonium salts being omitted (0·2 M-$NaHCO_3$, 100 ml.; 0·154 M-KCl, 4 ml.; 0·11 M-$CaCl_2$, 3 ml.; 0·154 M-KH_2PO_4, 1 ml.; 0·154 M-$MgSO_4,7H_2O$, 1 ml.; $Na_2S,9H_2O$, 0·02%. The solution was saturated with CO_2 after mixing, and then had pH 6·8).

Preparation of wheat-flour pentosan. Unbleached, untreated wheat flour (500 g.; supplied by Thomas Hulbert and Sons Ltd., New Era Flour Mills, Manchester) was shaken mechanically for 2 hr. with water (1 l.). The extract was separated by centrifuging, adjusted to pH 3 with HCl and filtered through glass wool to remove a little scum. Ethanol was added to the filtrate with stirring to a final concentration of 70% (v/v). The white precipitate was collected, and washed several times with ethanol, then with ether and dried *in vacuo*. The yield of crude pentosan was about 6 g. The ratio of pentosan:hexosan was approx. 1·5:1.

Crude pentosan (10 g.) was ground to a fine powder, mixed with water (300 ml.) and stored overnight in the refrigerator. Solution of still undissolved material was then completed by warming. To this was added human saliva (150 ml.) collected under toluene and centrifuged at 2500 *g* for 30 min. The mixture of pentosan and saliva was covered with toluene and incubated at 37° for 24 hr. with occasional shaking.

Trichloroacetic acid (10%, 400 ml.) was added to the mixture after incubation, and the flask stored at 0° overnight. The precipitate was removed by centrifuging and washed with a little cold 5% trichloroacetic acid solution. To the combined solution and washings (850 ml.) ethanol (2 l.) was added with stirring. The precipitate was washed with 70% (v/v) ethanol (3 × 150 ml.), then with absolute ethanol and finally with ether. The yield of pentosan was 4·35 g., i.e. 0·5% of the original flour [Found: N (Kjeldahl), 0·8%]. An acid hydrolysate, when tested chromatographically, was found to contain chiefly xylose and arabinose, in that order. Traces of glucose and galactose were also present. The hydrolysate contained 96% of reducing sugar, calculated as anhydropentose.

Preparation of rhodymenan. *Rhodymenia palmata* ('dulse'), as purchased in the Aberdeen market, was found to be almost free from other plants, and was used without sorting or cleaning. A quantity was obtained and stored at −20° until required. The thawed-out weed (1 kg.) was first passed through a kitchen mincer, and portions of the mince (100 g.) were mixed for 5 min. in a Waring Blendor with ice-cold 0·2 N-HCl (300 ml.). The extract was separated by a short centrifuging and filtered through glass wool into mechanically stirred acetone (2 vol.).

A further quantity of xylan was obtained from the acid-extracted weed by suspending in water (2·5 l.), neutralizing with 2N-NaOH and heating to 70° for 2 hr. The hot extract was separated by filtration at the pump, and the xylan precipitated with acetone. The crude materials obtained from the two extractions were combined, washed with acetone and then dissolved in 0·2N-NaOH (4 l.) by shaking. A little insoluble matter was removed by centrifuging, and the clear solution was mixed with Fehling's solution (400 ml.) with vigorous stirring. The gelatinous lump of copper complex was removed, washed twice by thorough kneading in water and dissolved by shaking in 0·2N-HCl (3 l.). The solution was poured into acetone (6 l.) and the white precipitate washed with acetone, first by decantation and finally on a Büchner filter until the washings were free from the orange-coloured complex copper ion. The yield was 25 g., equivalent to 2·5% of the wet weed. The copper content was 0·002%, the anhydroxylose content 100%; other sugars were absent.

Incubation of substrates with the bacterial suspension. The materials were ground to a fine powder and dissolved in a portion of the buffer solution by warming. The solution was mixed with the necessary volume of the bacterial suspension, covered with a thin layer of toluene and flushed with CO_2 to remove air. The stoppered vessel was incubated at 38° with occasional shaking. The quantity of bacterial suspension used, and the time of incubation in the different experiments, are mentioned in the appropriate places below. Samples withdrawn for reducing sugar determination were immediately deproteinized and reducing values determined, by the methods of Somogyi (1945*b*, *a* respectively). Results were expressed in terms of xylose. The bacterial suspensions were always used within 2 hr. of taking the rumen liquor from the sheep.

Chromatographic methods. Solutions which had been clarified as described in the preceding paragraph were de-ionized by passage through columns of Amberlite IR-120 (H) and IR-4B (OH) ion-exchange resins, or, with small samples, were treated in the tubes with small amounts of the two resins together. De-ionized samples which were too dilute were concentrated in a vacuum desiccator over anhydrous $CaCl_2$. Samples were applied to sheets of Whatman no. 1 paper and developed by the descending technique. The three solvent mixtures employed for development were: (*A*) ethyl acetate–pyridine–water (10:4:3, by vol.); (*B*) butanol–pyridine–water (10:3:3, by vol.); (*C*) upper layer of butanol–acetic acid–water (4:1:5, by vol.). The spray reagent was prepared by mixing equal volumes of 0·1M-aniline and 0·1M-phosphoric acid, both in water-saturated butanol. In order to separate oligosaccharides it was necessary to allow the solvent to run off the paper, so that R_F values could not be determined. R_x, the ratio of the distance travelled by the substance to the distance travelled by xylose on the same paper, was found to be a convenient value to measure. In this paper the values of R_x, for a substance in the three solvent mixtures (*A*), (*B*) and (*C*), are shown in that order.

Paper electrophoresis of oligosaccharides. Electrophoresis was carried out in 0·2M-borate buffer, pH 10, on Whatman no. 3MM paper, in an apparatus similar to that of Kunkel & Tiselius (1951). The applied p.d. was 230V, giving a current of 6–7 mA. The duration of electrophoresis was 5 or 18 hr. according to the mobility of the substances being examined. Substances were detected on the paper after drying, by the aniline phosphate spray. In calculating M_x (mobility with respect to xylose), correction was made for electroendosmotic flow by including *N*-2:4-dinitrophenylethanolamine as a coloured, non-mobile marker (Mould & Synge, 1954).

Large-scale hydrolysis of wheat-flour pentosan by rumen bacteria. This was carried out according to the general procedure described above. The buffer solution (450 ml.) contained wheat-flour pentosan (9·4 g.) and the bacteria from 800 ml. of rumen liquor. After incubation for 6 hr., the reducing value of the mixture corresponded to about 45% hydrolysis. The reaction was arrested and the mixture clarified, by the addition of 5% $ZnSO_4$ solution (50 ml.) followed by hot saturated $Ba(OH)_2$ until no more precipitate formed. Centrifuging and filtration yielded a clear, almost colourless solution which was de-ionized and concentrated *in vacuo* to 50 ml. This was added to the top of a column (400 mm. × 37 mm.) made from a mixture of equal parts of charcoal (B.D.H., acid-washed for decolorizing purposes) and Celite 535 (Johns Manville Co., New York) (Whistler & Durso, 1950). Aqueous ethanol was used as eluent, the concentration of ethanol being increased stepwise as required. The eluate was collected in fractions of 100 ml. each. Each fraction was tested chromatographically and also for total pentose by a modification of Drury's (1948) method. Appropriate fractions of the eluate were combined, concentrated *in vacuo*, filtered through a grade 5 sintered-glass filter, and dried. A portion of each of the combined fractions was hydrolysed by N-H_2SO_4 for 1 hr. at 100° and the neutralized hydrolysates were examined chromatographically.

Large-scale hydrolysis of rhodymenan. The xylan (5 g.) and a bacterial suspension prepared from 500 ml. of rumen liquor were incubated together in buffer solution (250 ml.). The treatment of the hydrolysate was the same as described for the wheat-flour pentosan.

Determination of molecular weights of oligosaccharides. Substances to be tested were dried *in vacuo* at 100° and the molecular weights determined by the hypoiodite method (Hirst, Hough & Jones, 1949).

Periodate oxidation of oligosaccharides. Quantities of approx. 2 mg. of the substances were oxidized in Warburg respirometers by the method of Perlin (1954) at a temperature of 18° and pH 5·7. The solutions remaining after titrating periodate were de-ionized and hydrolysed as described by Bishop (1956). The hydrolysates were tested for pentose both quantitatively (Drury, 1948) and qualitatively (paper chromatography in solvent mixture *B*).

Acid hydrolysis of carbohydrates. Complete hydrolysis was achieved by heating the substances with N-H_2SO_4 in sealed tubes in boiling water for 1 or 2 hr. as required. Partial hydrolysis was achieved by similar treatment with 0·1N-H_2SO_4 for 0·5 or 1 hr. Acid was neutralized with $BaCO_3$ before the hydrolysates were chromatographed.

EXPERIMENTAL AND RESULTS

Tests for oligosaccharide synthesis by the bacterial suspension. The bacterial suspension was incubated under the conditions described above in a solution containing 0·3% each of L-arabinose and D-xylose. Samples removed at intervals during 24 hr. and examined chromatographically were found to contain no sugars other than arabinose and xylose. In a similar experiment with xylobiose (see below) no sugars other than the starting material and xylose were detected after incubation.

Complete hydrolysis of the polysaccharides by the bacterial suspension. Solutions of the wheat-flour pentosan or rhodymenan (0·5%) were incubated with the bacterial suspension. After 18 hr. the reducing value in each case was the same as that of an equivalent amount of polysaccharide after complete acid hydrolysis (2 hr.). That hydrolysis by the bacterial suspension was almost complete was confirmed by chromatography; the wheat-flour pentosan hydrolysate contained arabinose, xylose and a barely detectable trace of xylobiose; the rhodymenan hydrolysate contained, in addition to xylose, barely detectable traces of xylobiose and 3^2-β-xylosylxylobiose (see below). For an explanation of this terminology see *Biochem. J.* (1957), **65**, 8.

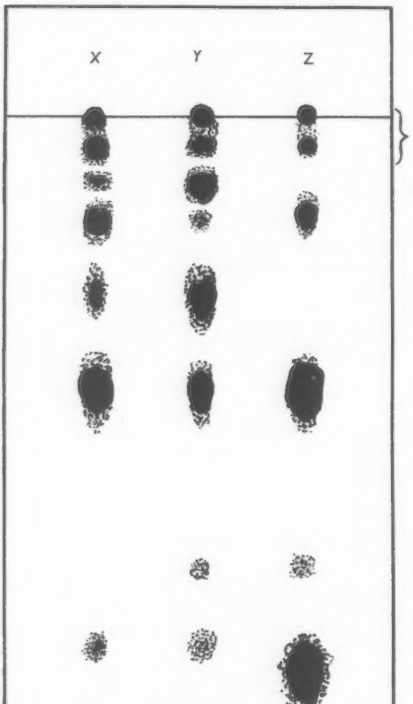

Fig. 1. Paper chromatogram of partially hydrolysed pentosans. *X*, Rhodymenan, 0·1 N-H_2SO_4; *Y*, rhodymenan, rumen bacteria; *Z*, wheat-flour pentosan, rumen bacteria. Solvent mixture *B*. Key to spots: (*a*) xylose; (*b*) arabinose; (*c*) rhodymenabiose; (*d*) glucose (from bacterial cells by autolysis); (*e*) xylobiose; (*f*) 3^2-β-xylosylxylobiose; (*g*) xylotriose; (*h*) xylose tetrasaccharide containing 1:3 linkage; (*i*) xylotetraose and higher oligosaccharides.

Course of hydrolysis of wheat-flour pentosan by the bacterial suspension. In order to follow the course of the hydrolysis in detail, wheat-flour pentosan (0·5%, w/v soln.) was incubated with the bacterial suspension and samples taken at frequent intervals were examined chromatographically. The first low-mol.wt. substance formed was arabinose, followed by the lower xylose oligosaccharides and finally by xylose itself (Fig. 1, *Z*). As hydrolysis proceeded the intensity of the colour reaction of the spots on the starting line increased to a maximum and then declined, concurrently with formation of appreciable amounts of xylose. Except in the final stages of the hydrolysis, arabinose was by far the most abundant product of low mol.wt. present. A chromatogram of rhodymenan after partial hydrolysis by rumen bacteria is shown in Fig. 1, *Y*. Comparison with Fig. 1, *Z* shows that the rhodymenan hydrolysate contains a number of substances not found in the wheat-flour pentosan hydrolysate. Thus although the spot (*c*) is hardly to be distinguished from arabinose it must represent another sugar, as arabinose is not a constituent of rhodymenan. Similarly, the spots (*f*) and (*h*) have no counterpart in the hydrolysate of the wheat-flour pentosan.

Oligosaccharides from large-scale hydrolysate of wheat-flour pentosan

Fractionation of the partial hydrolysate on a charcoal column yielded 12 fractions, as shown in Table 1.

Fraction 1. This was a syrup, which was shown by paper chromatography to contain a large proportion of arabinose, rather less xylose and a trace of glucose. On long standing it partly crystallized. The solid product was thoroughly drained on a porous tile and recrystallized from ethanol. It had m.p. 155°, not depressed on admixture with authentic L-arabinose, m.p. 158°.

Fraction 2. This was a syrup which did not crystallize until after seeding with crystals of authentic xylobiose. Recrystallized from aq. ethanol, the substance had m.p. 185°, $[\alpha]_D^{20}$ − 25° in water [Whistler & Tu (1951) found m.p. 187°, $[\alpha]_D$ − 25·5°] (Found: mol.wt. 298, 299. Calc. for $C_{10}H_{18}O_9$: 282). R_x values: 0·83, 0·39, 0·33.

Fraction 3. This was a syrup which could not be crystallized, but was chromatographically homogeneous; R_x values: 0·55, 0·12, 0·09, chromatographically indistinguishable in all three solvent systems from authentic xylotriose, and having $[\alpha]_D^{20}$ − 45° in water [Whistler & Tu (1951) found − 47° and Bishop (1955) found − 44·4°.] (Found: mol.wt. 426. Calc. for $C_{15}H_{26}O_{13}$: 414).

Fraction 4. This was a syrup containing a little xylotriose and a major, less-mobile component (R_x values: 0·28, 0·03, 0·03), very probably xylotetraose.

Fractions 5 *and* 6. These contained, in addition to the triose and tetraose of the preceding fractions, small and moderate amounts respectively of a less-mobile constituent (R_x values: 0·15, 0·02, 0·01), very probably xylopentaose.

Oligosaccharides from large-scale hydrolysate of rhodymenan

Charcoal-column separation of the hydrolysate yielded eight fractions, as shown in Table 2.

Table 1. *Charcoal-column fractionation of wheat-flour pentosan (9·4 g.) after partial hydrolysis by rumen bacteria in the presence of toluene*

Key to symbols in column 5: + + + + +, more arabinose than xylose; + + + +, almost as much arabinose as xylose; + + +, moderate proportion of arabinose; + +, trace of arabinose; +, barely detectable trace of arabinose; 0, no arabinose.

Fraction no.	Concn. of ethanol (%, v/v) in eluting agent	Vol. of eluate (l.)	Wt. of fraction (g.)	Arabinose content of hydrolysed fraction
1	0	2·3	4·39	+ + + + +
2	5	1·8	0·60	0
3	10	4·6	0·43	0
4	18	1·3	0·12	+
5	21	1·3	0·17	+
6	21	1·1	0·26	+
7	24	1·4	0·21	+ +
8	24	2·1	0·21	+ + +
9	27	1·1	0·14	+ + +
10	27	1·2	0·12	+ + +
11	30	3·8	0·32	+ + +
12	30	3·7	0·82	+ + + +
		Total	7·79 (83%)	

Table 2. *Charcoal-column fractionation of rhodymenan (5 g.) after partial hydrolysis by rumen bacteria in the presence of toluene*

Fraction no.	Concn. of ethanol (%, v/v) in eluting agent	Vol. of eluate (l.)	Wt. of fraction (g.)
1	0	1·2	2·20
2	5	1·0	0·30
3	10	0·5	0·07
4	10	0·6	0·17
5	10, 15	2·1	0·31
6	18	2·2	0·31
7	24	2·0	0·09
8	30–50	4·0	0·33
		Total	3·78 (76%)

Fraction 1. This was a syrup which readily crystallized. Recrystallized from ethanol with charcoal treatment, it had $[\alpha]_D^{20}$ +19° in water (*c*, 3·5), m.p. 148°, not depressed on admixture with authentic D-xylose, m.p. 147°.

Fraction 2. This was a syrup, which did not crystallize until seeded with authentic xylobiose. It crystallized from aq. ethanol as needles, m.p. 190°, $[\alpha]_D$ −25·8° in water; R_x values: 0·82, 0·39, 0·35; M_x value: 0·16 (Found: mol.wt., 286, 299. Calc. for $C_{10}H_{18}O_9$: 282). Complete acid hydrolysis yielded only xylose, and chromatograms of partial acid hydrolysates showed no sugars other than the starting material and xylose.

Fraction 3. The small amount of syrup partially crystallized in needles on standing and was purified by extraction with ethanol. *Rhodymenabiose* had $[\alpha]_D^{22}$ −18·4±0·6° in water (*c*, 3·25); R_x values: 1·05, 0·77, 0·69; M_x value: 0·60 (Found: C, 42·0; H, 6·7; mol.wt. 300. $C_{10}H_{18}O_9$ requires C, 42·55; H, 6·4%; mol.wt. 282). Complete acid hydrolysis yielded only xylose, and partial hydrolysis no sugars other than xylose and the starting material.

Fraction 4. This fraction, which did not crystallize, was a mixture of two substances. The minor component, which had R_x 0·14 in solvent mixture *B*, and M_x 0·02, was indistinguishable from authentic xylotriose. The major component was indistinguishable chromatographically from the trisaccharide found in fraction 5 (see below), but was differentiated from this substance by its higher electrophoretic mobility, the M_x value being 0·5. This fraction has not been examined further.

Fraction 5. This material, originally a colourless syrup, homogeneous both chromatographically and electrophoretically, crystallized on storage. Recrystallization from 85% ethanol yielded the *trisaccharide* as clusters of needles, m.p. 225° decomp., $[\alpha]_D^{22}$ −52°→ −47±1° in 1 hr. in water (*c*, 3·5); R_x values: 0·72, 0·26, 0·20; M_x value: 0·2 (Found: C, 43·2; H, 6·5; mol.wt. 408, 408. $C_{15}H_{28}O_{13}$ requires C, 43·5; H, 6·3%; mol.wt. 414).

Fraction 6. This material, apparently a tetrasaccharide, was chromatographically homogeneous but could not be made to crystallize. The tetrasaccharide had $[\alpha]_D^{21}$ −56·7±1° in water (*c*, 4·3); R_x values: 0·63, 0·08, 0·06; M_x value: about 0·02 (Found: mol.wt., 526, 538. Calc. for $C_{20}H_{34}O_{17}$: 546).

Examination of the new trisaccharide

Periodate oxidation. The trisaccharide (1·95 mg.) was submitted to oxidation by sodium periodate. Oxidation was complete in 24 hr., at which time the CO_2 liberated from solution corresponded to 2·85 mol.prop. of formic acid, and the periodate consumed was 4·04 mol.prop. These figures are the means of three determinations. Xylose (1·81 mg., equivalent to 85% of the theoretical value for one mol.prop.) was the only sugar found in the hydrolysate of the product from the three combined oxidations.

Decomposition of the triosazone by ion-exchange resin. The trisaccharide (17 mg.) was heated in boiling water for 30 min. with hydrated sodium acetate (54 mg.), phenylhydrazine hydrochloride (30 mg.) and water (0·2 ml.). The precipitate of fine orange needles which separated on chilling was collected by centrifuging and dissolved in ethanol. This solution was treated with Zeo-Karb 215 as described by Finan & O'Colla (1955). More satisfactory chromatograms of the sugar in the eluate from the resin were obtained by first evaporating the solution to dryness *in*

vacuo, extracting the residue with water, and passing through a small column (20 mm. × 8 mm.) of charcoal–Celite. When this column was washed with increasing concentrations of ethanol in water, only the 5% ethanol eluted any sugar. The substance had R_x values: 1·05, 0·77, 0·67, indistinguishable from rhodymenabiose.

Acid hydrolysis. Complete hydrolysis (2 hr.) yielded only xylose. Partial hydrolysis (0·5 hr.) yielded, in addition to the starting material and xylose, two other substances, R_x (in solvent mixture *B*) 0·38 and 0·77, the former being the stronger. These corresponded to xylobiose and rhodymenabiose respectively, when run on the same paper.

Examination of the new tetrasaccharide

Periodate oxidation. Oxidation of the tetrasaccharide (2·29 mg.) under the conditions described was complete in 27 hr., when formic acid production and periodate consumption were respectively 2·86 and 4·95 mol.prop. (means of two determinations). Xylose (0·91 mg., equivalent to 72% of the theoretical value for 1 mol.prop.) was the only sugar found in the hydrolysate of the oxidation product.

Decomposition of the tetraosazone by ion-exchange resin. The tetrasaccharide was converted into the osazone as described above. As nothing separated when the reaction mixture was cooled, it was diluted with ethanol and the whole transferred to the Zeo-Karb 215 column. When the decomposition product was placed on a small charcoal–Celite column it was not eluted by water or 5% (v/v) ethanol, but was eluted by 15% (v/v) ethanol. It had R_x values 0·7, 0·24, 0·22, and M_x value 0·5.

Acid hydrolysis. Complete hydrolysis yielded only xylose, whereas partial hydrolysis yielded three substances in addition to unchanged starting material and xylose. In order of decreasing yield, they had R_x values (in solvent *B* only) 0·39, 0·75 and 0·21, indistinguishable respectively from xylobiose, rhodymenabiose and the trisaccharide found in fraction 5.

Acid hydrolysis of rhodymenan

In Fig. 1, *X* is shown a chromatogram of a partial acid hydrolysate of rhodymenan (1 hr.). Comparison with the result of a corresponding hydrolysis by rumen bacteria (Fig. 1, *Y*) shows that, at least for the lower oligosaccharides, the same substances are present in both, although in different proportions. Thus whereas the acid hydrolysate contains more xylotriose than the trisaccharide found in fraction 5, the positions are reversed in the bacterial hydrolysate. Similarly, the acid hydrolysate contains a lesser proportion of the tetrasaccharide found in fraction 6 than does the bacterial hydrolysate.

DISCUSSION

Of the two types of linkage in the wheat-flour pentosan molecule, that between arabinose and xylose seems to be more easily broken by the suspension of rumen bacteria than that between xylose and xylose. For this reason, no mixed arabinose and xylose oligosaccharides were found in the di- and tri-saccharide fractions, and only traces in the tetra- and penta-saccharide fractions. The ease with which the arabinose side chains are split-off by the bacterial suspension parallels the lability of the arabofuranoside linkage to acid hydrolysis. Perlin (1951*b*) found that more than three-quarters of the arabinose in the pentosan could be removed under mild acid conditions, which left the xylan chain almost intact. Decomposition of the xylan chain by the rumen bacteria proceeds by apparently random hydrolysis, and not by stepwise attack at the ends of the chain, as is shown by the presence in a partial hydrolysate of a series of higher oligosaccharides (Table 1).

The xylose oligosaccharides obtained from the wheat-flour pentosan in the present work belong to the homologous β-1:4 series. They were first isolated by Whistler & Tu (1951, 1952, 1953) from a partial acid hydrolysate of maize-cob xylan, and later from wheat-straw hemicellulose by Bishop (1955). No oligosaccharides have hitherto been reported from hydrolysates of wheat-flour pentosan.

The chief feature of interest in the experiments on rhodymenan is the production of a number of new xylose oligosaccharides during the hydrolysis by rumen bacteria. These substances were absent from similar hydrolysates of the wheat-flour pentosan (Fig. 1, *Y*, *Z*), in which only known substances have so far been detected. It may be noted, before proceeding to consider the new oligosaccharides, that, in addition to xylose itself, xylobiose and xylotriose are present in the hydrolysate of rhodymenan. The presence of xylotetraose and higher members of the β-1:4 series has not yet been established.

The absence of any synthesis when the bacterial suspension was incubated with arabinose, xylose or xylobiose seems to exclude the possibility that these new oligosaccharides arose by transglycosylation. The occurrence of the new xylose oligosaccharides in a dilute solution of rhodymenan after partial acid hydrolysis (Fig. 1, *X*) is further evidence that they are genuine fragments of the polysaccharide, and are not products of enzymic synthesis. Since the polysaccharide possesses the unique feature of having xylose β-1:3 linkages, these new oligosaccharides must be distinguished from those of the β-1:4 series by their having one or more β-1:3 linkages.

Rhodymenabiose. The low yield of this substance from the hydrolysate of rhodymenan has not allowed a complete investigation of its chemical properties. The formation of xylose only on complete hydrolysis, and the molecular weight as determined by hypoiodite oxidation, show the substance to be a xylose disaccharide. In agreement with this is the observation that during hydrolysis by dilute acid no sugars are detectable chromatographically other than rhodymenabiose and xylose. In the light of what is known of the structure of the parent polysaccharide, rhodymenabiose must therefore be *O*-β-D-xylopyranosyl-(1 → 3)-D-xylopyranose. An interesting feature of

the substance is its high mobility on the paper chromatogram. In solvent mixtures *B* and *C* it is twice as mobile as xylobiose ($R_{xylobiose}$ values, 1·97, 1·98 respectively), and in solvent mixture *A* it is more mobile than xylose itself (R_x value, 1·05). This is the first instance known to the author of a disaccharide having a higher chromatographic mobility than the parent monosaccharide.

The trisaccharide. This substance was obtained in approximately 5% yield from the xylan. Elementary analysis and hypoiodite oxidation showed it to be a trisaccharide, $C_{15}H_{26}O_{13}$. Assuming only that the molecule consists of three β-xylopyranose units, it is possible from the results of periodate oxidation and other experiments to assign a structure without reference to the parent polysaccharide. One mole of the substance, when oxidized by periodate, consumed four moles of oxidant, yielding three moles of formic acid, and one mole of xylose remained intact in the oxidation product. Of the 12 possible trisaccharides, only *O*-β-D-xylopyranosyl-(1 → 3)-*O*-β-D-xylopyranosyl-(1 → 4)-D-xylopyranose (3^2-β-xylosylxylobiose) would be expected to give these proportions on oxidation. Confirmation that the new trisaccharide has this structure is provided by the results of partial hydrolysis, both of the substance itself by dilute acid, and of the osazone by ion-exchange resin. In the former case two intermediate products of hydrolysis are detectable in addition to xylose, indistinguishable chromatographically from xylobiose and rhodymenabiose. Finan & O'Colla (1955) examined the effect of an acidic ion-exchange resin under controlled conditions on osazones of oligosaccharides. In each case, only the glycosidic linkage nearest to the osazone grouping was hydrolysed, yielding the osazone of a monosaccharide and a reducing sugar containing one monosaccharide unit less than the original oligosaccharide. Applying this technique to the trisaccharide, the substance formed by action of the resin was chromatographically indistinguishable from rhodymenabiose. The results of these two hydrolytic experiments are explicable only if the trisaccharide is 3^2-β-xylosylxylobiose, confirming the conclusion drawn from the periodate oxidation. The structure advanced for the trisaccharide is, of course, in agreement with what is already known of the structure of rhodymenan.

3^2-β-Xylosylxylobiose resembles rhodymenabiose in being much more mobile on a paper chromatogram than the corresponding oligosaccharide containing only 1:4 linkages, the $R_{xylotriose}$ values being 1·36, 1·94 and 1·43 respectively in the three solvent mixtures.

The tetrasaccharide. This material, although chromatographically pure, has not yet been obtained in crystalline form. The complete hydrolysis of the material yielding only xylose, and the molecular weight as determined by hypoiodite oxidation, show it to be a xylose tetrasaccharide.

Periodate oxidation of the tetrasaccharide yielded on a molar basis 3 moles of formic acid, consumed 5 moles of periodate, and left 1 mole of xylose intact in the oxidized product. Of the eight possible linear β-xylopyranose tetrasaccharides containing only 1:3 or 1:4 linkages or both, only two would be expected to give these proportions on oxidation. These are (*a*) *O*-β-D-xylopyranosyl-(1 → 4)-*O*-β-D-xylopyranosyl-(1 → 3)-*O*-β-D-xylopyranosyl-(1 → 4)-D-xylopyranose, i.e. 3^2-β-xylobiosylxylobiose, and (*b*) *O*-β-D-xylopyranosyl-(1 → 3)-*O*-β-D-xylopyranosyl-(1 → 4)-*O*-β-D-xylopyranosyl-(1 → 4)-D-xylopyranose, i.e. 3^3-β-xylosylxylotriose. A choice between these cannot be made with certainty on the evidence available at present. Structure (*a*), however, seems rather more probable, for the following two reasons. (1) Xylotriose is apparently not formed during partial hydrolysis of the tetrasaccharide. (2) Hydrolysis of the osazones of (*a*) and (*b*) according to the procedure of Finan & O'Colla (1955) should yield respectively 4^2-β-xylosylrhodymenabiose and 3^2-β-xylosylxylobiose, i.e. the trisaccharide already found in the bacterial hydrolysate of rhodymenan. The product formed in this reaction from the osazone of the tetrasaccharide cannot be 3^2-β-xylosylxylobiose, because of the widely differing electrophoretic mobilities of the two substances.

Like rhodymenabiose and the new trisaccharide, the tetrasaccharide has a higher chromatographic mobility than the corresponding oligosaccharide containing only 1:4 linkages; the $R_{xylotetraose}$ values in the three solvent mixtures are 2·2, 2·0 and 2·1.

When $\log(1/R_F - 1)$ (Bate-Smith & Westall, 1950) of the oligosaccharides present in the partial hydrolysate of rhodymenan, calculated from a chromatogram in solvent *B*, is plotted against the degree of polymerization, the points fall on two nearly parallel straight lines (Fig. 2). The upper line represents the oligosaccharides containing only 1:4 linkages, the lower one those oligosaccharides containing one 1:3 linkage. It is also of interest to note that rhodymenabiose and the new trisaccharide are more firmly held on a charcoal column than are the corresponding oligosaccharides containing only 1:4 linkages.

The value of establishing structures of oligosaccharides as a means to understanding the nature of the polysaccharides from which they are produced has become widely recognized recently (see, for example, Hirst, 1955). The present investigation therefore might possibly shed light on the structure of rhodymenan. The isolation in substantial yield of a pure crystalline trisaccharide

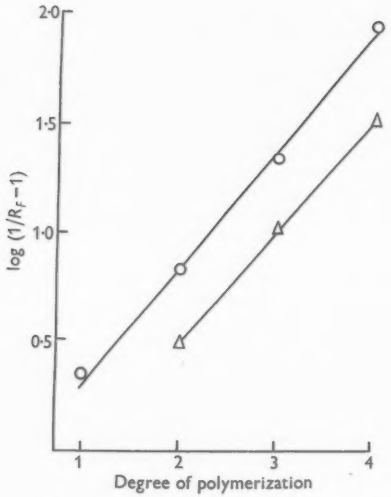

Fig. 2. Relationship between chromatographic mobility and degree of polymerization of xylose oligosaccharides. ○, β-1:4 series; △, oligosaccharides from rhodymenan, each containing one β-1:3 linkage. Solvent mixture *B*. R_F was calculated from R_x multiplied by R_F of xylose (0·31).

containing both a 1:3 and a 1:4 linkage affords conclusive proof that the polysaccharide molecule does indeed contain both types of linkage. This conclusion had been previously drawn from the failure of all attempts to separate the xylan into an all 1:4 fraction and an all 1:3 fraction. No substances were detected in the hydrolysate which could be the trisaccharide 3^2-β-xylosylrhodymenabiose or the tetrasaccharides 3^2-β-xylobiosylrhodymenabiose or 3^2-β-rhodymenabiosylxylobiose. The conclusion may thus be tentatively drawn that the rhodymenan chain does not contain two adjacent 1:3 linkages.

In contrast to the hydrolysis of the wheat-flour pentosan, which proceeds in a similar fashion whether carried out by acid or by the rumen bacteria, the hydrolysis of rhodymenan by these two agencies shows some qualitative differences (Fig. 1, *X* and *Y*). The total proportion of substances containing the 1:3 linkage (spots *c*, *f* and *h*) appears to be greater in the bacterial hydrolysate, suggesting that the relative susceptibility to hydrolysis of the two types of linkage varies according to the hydrolytic agent. Duncan, Manners & Ross (1954, 1956) suggested that when attacked by the enzymes of *R. palmata* itself, rhodymenan splits rather more readily at the 1:3 linkage.

SUMMARY

1. The hydrolysis of two water-soluble pentosans by toluene-treated suspensions of bacteria from sheep rumen has been studied.

2. The pentosan of wheat flour and the xylan of the red seaweed *Rhodymenia palmata*, for which the trivial name rhodymenan is proposed, are both hydrolysed to completion by the bacterial suspension under physiological conditions.

3. Hydrolysis of the wheat-flour pentosan by the bacterial suspension proceeds by removal of the arabinose side chains and fragmentation of the main xylose chain simultaneously. Partially hydrolysed pentosan was fractionated on a charcoal column, yielding arabinose and xylose, xylobiose, xylotriose, xylotetraose and xylopentaose, together with a series of higher oligosaccharides. Oligosaccharides of low molecular weight, containing both arabinose and xylose, are present in the hydrolysate only in traces.

4. Rhodymenan, when partially hydrolysed by the bacterial suspension, yields xylose, xylobiose and xylotriose, together with a number of hitherto unknown xylose oligosaccharides. Two of the latter have been obtained in crystalline form; one is a disaccharide for which the name *rhodymenabiose* is proposed and the other is a trisaccharide. An amorphous xylose tetrasaccharide has also been isolated.

5. The structure *O*-β-D-xylopyranosyl-(1 → 3)-D-xylopyranose is suggested for rhodymenabiose. The structure of the trisaccharide has been shown to be *O*-β-D-xylopyranosyl-(1 → 3)-*O*-β-D-xylopyranosyl-(1 → 4)-D-xylopyranose. The sequence of linkages in the tetrasaccharide is either β-1:4, β-1:3, β-1:4 or, less probably, β-1:3, β-1:4, β-1:4.

6. The structure of rhodymenan is discussed in the light of these findings.

The author gratefully acknowledges the encouragement and advice of Dr A. E. Oxford during the course of this work. Dr G. O. Aspinall, of the Department of Chemistry, University of Edinburgh, kindly supplied samples of crystalline xylobiose and of xylotriose. Dr A. G. Ross of the same laboratory gave useful advice on the preparation of rhodymenan. Thanks are also due to my colleagues, Dr R. L. M. Synge for advice on paper electrophoresis and Dr C. F. Mills for a copper determination, and to Mr H. Stewart for much experimental assistance.

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The Purification and Properties of Rabbit-Muscle Myokinase

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Previous methods for purifying myokinase (adenylate kinase) have made use of the procedure of Colowick & Kalckar (1943), which has been improved by Noda & Kuby (1955, 1956), Bendall (1954) and Bowen & Kerwin (1955). A different method, to be described here, yields a preparation which appears to be very similar in its physical properties to the crystalline enzyme of Noda & Kuby (1956). A study has also been made of the properties of the sulphydryl groups of the enzyme and of its heat stability.

Preliminary reports of this work have been given elsewhere (Callaghan, 1956, 1957).

EXPERIMENTAL

Materials and methods

Adenosine triphosphate (*ATP*). This was obtained as the disodium salt from the Sigma Chemical Co., St Louis, Mo., U.S.A.

Adenosine diphosphate (*ADP*). This was prepared by the dephosphorylation of ATP by means of the potato adenyl pyrophosphatase of Lee & Eiler (1951), and the barium salt was prepared according to Le Page (1951). This was stored at −15°. Chromatographic analysis indicated that 94·5% of the phosphate was present in the form of ADP, 1% as adenosine 5′-monophosphate (AMP) and 4·5% as inorganic phosphate.

Adenosine 5′-monophosphate. AMP was obtained from Roche Products Ltd., Welwyn Garden City, England. Solutions of AMP were brought to pH 7 before use.

Sodium creatine phosphate hexahydrate. This was a gift from the Sigma Chemical Co.

L-*Cysteine hydrochloride.* The preparation (British Drug Houses, London) was dissolved in water and carefully neutralized to pH 7.

Sodium p-*chloromercuribenzoate.* This was a gift from Dr G. Weber.

Methylmercury nitrate. This was prepared from methylmercuric iodide by treatment with silver nitrate in ethanol (Johns, Peterson & Hixon, 1930). The product was twice recrystallized from water, and had a melting point of 59–60°. Methylmercury iodide was prepared from mercury and methyl iodide by the photochemical method of Maynard (1932), and, after two crystallizations from alcohol, had a melting point of 144°. The combined methods gave a yield of 70% of methylmercury nitrate.

Haemoglobin and cytochrome c. These were gifts of Dr F. W. J. Teale.

Hexokinase and glucose 6-*phosphate dehydrogenase.* A mixture of these enzymes ('coupling enzyme') was obtained from yeast by the method of Oliver (1955). The freeze-dried preparation was stored in the refrigerator.

Crystalline bovine-plasma albumin. This was obtained from Armour Laboratories, Lindsey St., London, E.C. 1.

Spectrophotometer. The Unicam spectrophotometer model SP. 500 was employed for the determination of optical density. At 340 mμ, glass cuvettes (1 cm.) were used and quartz cuvettes (1 cm.) were used to measure optical density at 260 and 280 mμ.

In assaying enzyme activity, the temperature of the cuvettes was controlled by a water-jacketed cell compartment made according to Campbell & Simpson (1953).

Routine assay of myokinase. The enzyme was assayed by the method of Oliver (1955). This method gave duplicate results which agreed within ±5% of each other, and was invaluable for the rapid assay of the enzyme in any large series of experiments. The method depends on the reactions:

2ADP→ATP + AMP; ATP + glucose→ADP + glucose 6-phosphate; glucose 6-phosphate + oxidized triphosphopyridine nucleotide (TPN)→6-phosphogluconic acid + reduced TPN.

The rate of reduction of TPN was measured by observing the increase in optical density at 340 mμ with time. The following constituents were added to a test cuvette: 2·8 ml. of 0·075 M-sodium diethyl barbiturate–HCl buffer, pH 8·6, containing 0·1 M-KCl, 0·01 M-$MgCl_2$ and TPN (3 mg./20 ml.); 0·2 ml. of 0·02 M-ADP solution; 0·2 ml. of 0·5 M-glucose; 0·2 ml. of 'coupling enzyme' soln. (20 mg./ml. in barbitone buffer). On addition of the coupling enzyme, a small 'blank' rate of extinction increase was observed. Suitably diluted myokinase in cysteine solution (0·1 ml.) was added, and the rate measured at 30 sec. intervals. All readings were measured against a blank cuvette containing 0·2 ml. of coupling enzyme soln. and 2·8 ml. of buffer. The steady rate of reaction was obtained from a plot of optical density against time. At 30°, a rate of increase of extinction at 340 mμ of 0·1/min. (corrected for blank activity) under the above conditions was arbitrarily chosen as the unit of enzyme activity. It was found that, at this velocity, the coupling enzymes were in sufficient excess to ensure that the myokinase reaction was the rate-determining factor.

Chromatographic assay. A more precise measurement of enzyme activity was performed as follows: the enzyme was activated with cysteine, as described below, and added to a buffered solution containing Mg^{2+} ions and the requisite concentration of adenosine phosphates at 30°. After incubation for 1 min., and at subsequent times, samples of this mixture were treated with 0·1 vol. of 30% (w/v) trichloroacetic acid and cooled to 0°.

These samples, which could be safely stored at −15°, were then analysed chromatographically to separate ATP, ADP, AMP and inorganic phosphorus by the method of Krebs & Hems (1953); the individual nucleotides on chromatograms were wet-ashed, and the phosphorus content was determined by the method of Berenblum & Chain (1938).

The following digestion mixture was found to ash the paper with great rapidity: 1·25 g. of ammonium molybdate (AnalaR) in 10 ml. of water was added to 50 ml. of H_2SO_4–$HClO_4$ (3:2, v/v). On standing overnight, a crystalline precipitate settled. A portion (0·5 ml.) of the supernatant was used to ash each specimen.

Protein estimation. The protein concentration in enzyme solutions was determined both spectrophotometrically and by measurements of dry weight.

For the determination of protein concentration in the presence of nucleotides, the approximate formula of Kalckar (1947) was applied: $1{\cdot}45.E\,(280\ m\mu) - 0{\cdot}74.E\,(260\ m\mu)$ = mg. of protein/ml. (in a 1 cm. cuvette). Purified myokinase was dialysed exhaustively against water, and spectrophotometric and dry-weight determinations showed that the enzyme gave an optical density, $E^{1\%}_{1\ cm.}$ (280 mμ) = 11·1.

Electrophoresis. The isoelectric point of the purified enzyme was determined by paper electrophoresis in 0·03 M-phosphate buffers, with an EEL apparatus.

Myokinase was also analysed in the zone electrophoresis apparatus of Flodin & Porath (1954). A buffer of 0·0115 M-borate and 0·0155 M-NaH_2PO_4, pH 8·4 (I 0·05) was used. The column of the apparatus was packed with acetylated cellulose, prepared according to Campbell & Stone (1956).

Purification of myokinase. All operations were carried out in the cold. Fresh rabbit muscle was chilled in cracked ice and minced twice. The mince was extracted with 1·5–2 vol. of 0·05 M-KCl for 20 min., and the centrifuged extract was dialysed for 3 hr. against cold running water and then against 20 times its volume of distilled water at 4° for 6 hr. The fractionation of the dialysed extract followed the method of Askonas (1951). The extract was adjusted to pH 6·5 with $NaHCO_3$ and cooled in a stainless-steel vessel to 0° with constant stirring. Propanol was added in a fine jet at a rate compatible with the maintenance of low temperature. After 20% (v/v) has been added, the temperature was lowered to −5°, and the propanol added to a final concentration of 40% (v/v). After equilibration for 20 min. the protein precipitate was centrifuged at −5° in pre-cooled containers; the supernatant was discarded and the precipitate immediately suspended and broken up in ice-cold 0·033 M-phosphate buffer, pH 7·4, and dialysed against the same buffer for 3 hr. A flocculent precipitate appeared which contained the enzyme. This was collected and the supernatant discarded. The precipitate was suspended in cold water (0·25 vol./vol. of original extract), dissolved at pH 2 by the dropwise addition of 2 N-HCl and treated with 0·04 vol. of 5 M-NaCl, and readjusted to pH 6·5 with 2 N-NaOH, when a dense precipitate reappeared. The clear supernatant was a stable solution of the enzyme. The precipitate could again be extracted with HCl, as described above, for further enzyme. The combined solutions were fractionated on a column of calcium phosphate gel (Swingle & Tiselius, 1951), Whatman cellulose powder being used instead of the conventional inert packing material (Hyflo Supercel). A mixture of 4 parts of cellulose powder to 1 part of gel (dry wt.) was used. A column 15 cm. × 6 cm.² was adequate for the protein derived from 500 g. of muscle. The solution was driven into the column with O_2 at a pressure of 20 lb./in.² After the protein on the column had been washed with 0·2 M-NaCl to remove the bulk of nucleotides present, a single peak absorbing light at 280 mμ (see Fig. 1) was eluted with 0·025 M-phosphate buffer, pH 7, in 0·2 M-NaCl, and 14 ml. fractions were collected in polythene tubes. The enzyme peak was followed by a trail of inactive protein. Fractions which displayed enzymic activity were combined and fractionated with $(NH_4)_2SO_4$, the fraction which precipitated between 0·65 and 0·85 saturation being centrifuged down at 25 000 *g* and dissolved in 0·2 M-NaCl. Successive

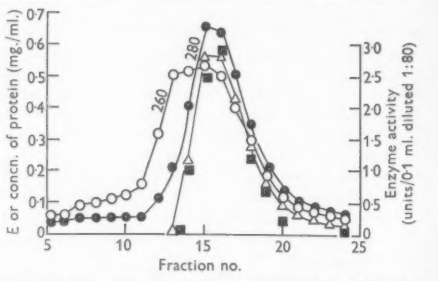

Fig. 1. Protein content and enzyme activity of fractions eluted from a column of calcium phosphate gel (see text). ○, Optical density at 260 mμ; ●, optical density at 280 mμ; △, protein concentration; ■, enzyme activity.

Table 1. *Details of a representative preparation of myokinase (see text)*

Treatment	Total units	Total protein (mg.)	Volume (ml.)
Extract from mince	1 550 000	8450	545
Dialysed extract	1 470 000	8250	610
Solution of enzyme in NaCl, obtained after propanol fractionation and subsequent treatment with HCl and NaOH	435 000	95	125
Further solution of enzyme in NaCl after re-extraction of precipitate with HCl	232 000	42·5	150
Combined fractions effluent from $Ca_3(PO_4)_2$ column	620 000	67·5	68
0·65–0·85 saturated $(NH_4)_2SO_4$. Enzyme dissolved in 0·2M-NaCl	605 000	55	10

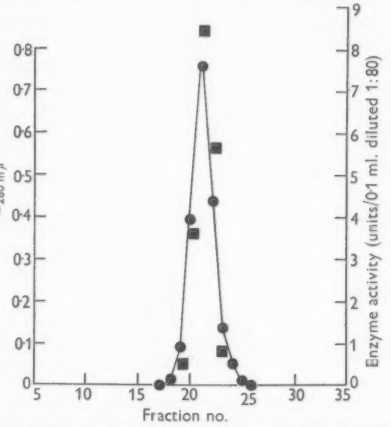

Fig. 2. Protein content and enzyme activity of 6 ml. fractions eluted from zone-electrophoresis apparatus after electrophoresis for 7 hr. 20 min. at 140 V D.C. at 5° in borate–phosphate buffer (0·0115M-$Na_2B_4O_7$, $10H_2O$–0·0155M-NaH_2PO_4; pH 8·4; I 0·05). ●, Optical density at 280 mμ; ■, enzyme activity.

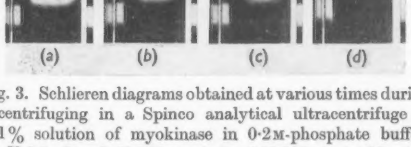

Fig. 3. Schlieren diagrams obtained at various times during centrifuging in a Spinco analytical ultracentrifuge of 1% solution of myokinase in 0·2M-phosphate buffer, pH 6·5. Speed, 50 730 rev./min. (186 000 *g*). (*a*) 32 min.; (*b*) 48 min.; (*c*) 64 min.; (*d*) 240 min.

preparations of myokinase were equally pure after this step. The activity and purity data for a representative preparation are given in Table 1.

Storage and dialysis of the enzyme. Solutions of myokinase in 0·2M-NaCl or neutral phosphate buffer are stable for several weeks at 2°, if kept in polythene tubes. For reasons which are dealt with below, glass vessels should not be used. The enzyme is also inactivated on dialysing in cellophan sacs. Bendall (1954) reported that myokinase could be dialysed, without loss of activity, in collodion tubes coated with haemoglobin. Collodion tubes prepared by the method of Adair (1925) as described by Gutfreund (1952) may be coated with cytochrome *c* or haemoglobin by contact of the inner surface of the tube with a 1–2% solution of either protein for 48 hr. Such coated tubes may be used for the dialysis of myokinase, with small loss of activity. Nine out of ten such tubes were found to be satisfactorily coated for the safe dialysis of myokinase.

RESULTS

Properties of myokinase

The myokinase preparation described here gave both biuret and nitroprusside reactions, indicating that it is a sulphydryl-containing protein. The enzyme is stable over the range pH 6–9. Its stability in acid is described more fully below.

Zone electrophoresis (Fig. 2) indicated that the preparation contained a single protein component. From electrophoresis on paper it was estimated that the enzyme is isoelectric at pH 4·3; this figure differs from the value pH 6·05 obtained by Noda & Kuby (1956) by an unspecified method, but as myokinase is partially inactivated during paper electrophoresis and tends to adhere to the paper, the value pH 4·3 should be regarded as a tentative one.

Myokinase appears to be free from bound nucleotides, the ultraviolet-light absorption having a ratio $E_{280\ m\mu}/E_{260\ m\mu} = 1{\cdot}28$. The optimum pH value for enzyme activity has not been determined precisely, but the highest activity was observed at pH 7·5. Over the range pH 7–8 the activity does not fall below 65% of the maximum.

Myokinase shows a single component in the ultracentrifuge (Fig. 3). The sedimentation coefficient of a 1% solution in water (S_{20}) was $1{\cdot}7 \times 10^{-13}$. Noda & Kuby (1956) report a value $S_{25} = 2{\cdot}3 \times 10^{-13}$ for the crystalline enzyme on 0·15M-KCl. Corrected for electrolyte, viscosity and temperature, this corresponds to a value S_{20}

(water) = $1{\cdot}8 \times 10^{-13}$. These same authors have assigned a molecular weight of 21 000 to the enzyme, based on sedimentation and diffusion measurements. The writer has found a unit molecular weight of approx. 10 800/sulphydryl group, from titration with methylmercury nitrate (see below); thus a molecular weight of 21 600 may be postulated.

Titration of the sulphydryl groups of myokinase. Sulphydryl groups were titrated with methylmercury nitrate by essentially the same method as that of Edelhoch, Katchalski, Maybury, Hughes & Edsall (1953). A solution of 5 M-guanidine hydrochloride was adjusted to pH 10 with solid anhydrous Na_2CO_3 and filtered. Ethylenediaminetetra-acetate was added to 0·01 M-concentration of this substance. To 2 ml. of the cold guanidine reagent 0·5–1 ml. of myokinase solution (5 mg./ml.) and 1 drop of fresh 10 % sodium nitroprusside were added, and the purple colour was titrated to a colourless end-point. The titration was carried out rapidly, near 0°, with approx. 10^{-3} M-methylmercury nitrate which had been standardized against freshly diluted thioglycollic acid. The thioglycollic acid (British Drug Houses Ltd., 97 % pure) was itself standardized by acidimetry against NaOH solution with thymolphthalein indicator.

Three portions of myokinase solution, each containing 5·4 mg. of enzyme, gave 0·500, 0·500 and 0·458 μmole of sulphydryl. For a molecular weight of 21 000 (Noda & Kuby, 1956) these quantities were equivalent to 1·94 and 1·78 SH groups/molecule. Conversely, a unit molecular weight of 11 000 ± 500/sulphydryl group could be estimated.

A further sample of enzyme, which had been treated with the guanidine reagent containing 10^{-3} M-*p*-chloromercuribenzoate, failed to give the nitroprusside reaction and no titration was possible.

Samples (0·5 ml.) of the dialysed muscle extract (see Table 1), which contained 14 mg. of protein/ml., contained 0·72–0·74 μmole of SH. Since the enzyme content of this extract (by assay) was not more than 3–4 % of the total protein, a 35–44-fold excess of sulphydryl groups over those possessed by myokinase was present.

Inhibition by p-*chloromercuribenzoate.* Equimolar concentrations of enzyme and *p*-chloromercuribenzoate showed approx. 40–50 % inhibition of enzyme activity. This inhibition, which is reversed by addition of cysteine, will be the subject of a further paper.

Ultracentrifuge studies. The possibility was entertained that oxidation of SH groups in myokinase might lead to disulphide linkages being formed between different molecules. This would lead to the appearance of polymers of higher molecular weight.

Schlieren diagrams, obtained in the ultracentrifuge, of the fully active enzyme and the enzyme after total oxidation (but not irreversible inactivation) by dialysis for 48 hr. in cellophan gave S_{20} values (water) of $1{\cdot}7 \times 10^{-13}$ in both cases. No additional components were detected for the oxidized enzyme.

Velocity of forward reaction. The speed of the forward reaction 2ADP → ATP + AMP decays rapidly with time (Fig. 4). By drawing tangents to the progress curve, by the mirror method (Joos, 1934),

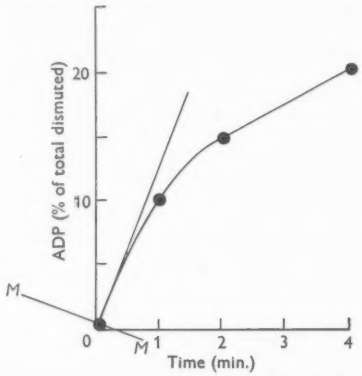

Fig. 4. Decay of velocity of reaction 2ADP → ATP + AMP at 30° with time (see text). At zero time, the reaction mixture consisted of 0·075 M-2-amino-2-hydroxymethyl-propane-1:3-diol (tris) buffer, pH 7·5, 0·0045 M-ADP. 0·002 M-$MgCl_2$, 0·006 M-cysteine hydrochloride and 1 μg of myokinase, in a total volume of 4·5 ml. Portions were withdrawn at various times and treated with 0·1 vol. of 30 % trichloroacetic acid. The line *MM* indicates the position of the mirror (see text).

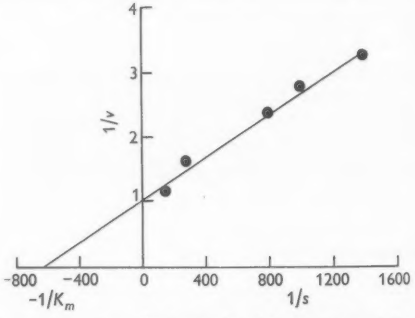

Fig. 5. Lineweaver–Burk plot for determination of K_m at 30° (see text). Myokinase (0·5 μg.) was used and the reaction stopped after 1 min. by treatment with 0·1 vol. of 30 % trichloroacetic acid. At zero time the reaction mixtures consisted of 0·075 M-tris buffer, pH 7·5, 0·002 M-$MgCl_2$, 0·006 M-cysteine hydrochloride. Total volume, 1·6 ml. Intercept at $-1/K_m$ indicates a K_m value of $1{\cdot}6 \times 10^{-3}$ M.

it can be shown that a tangent drawn to the curve at the point of 10% dismutation has a slope which is 56% of that of the initial tangent. The velocity falls to 22% when 15% of the ADP is dismuted. It is essential therefore to measure the rate of myokinase activity at very short time intervals before an appreciable fraction of the ADP is dismuted.

The velocity v determined graphically from the initial tangent in Fig. 4 was obtained with a substrate concentration of $4{\cdot}5 \times 10^{-3}$ M. The maximum velocity (V) was derived from this by the equation $V = v(K_m + [S])/[S]$. The K_m value (Fig. 5) is $1{\cdot}6 \times 10^{-3}$ M [this value for K_m, obtained by the graphical method of Lineweaver & Burk (1934), is similar to the figure $1{\cdot}3 \times 10^{-3}$ M reported by Siekevitz & Potter (1953) for the mitochondrial myokinase of rat liver]. Experimentally, $v = 1{\cdot}9$ μ-moles of ADP/μg. of protein/min. at pH 7·5 and 30°. Therefore $V = 2{\cdot}5 \times 10^5$ moles of ADP/10^5 g. of protein/min. With an enzyme molecular weight of 21 000 the turnover number is therefore 52 500, or for a molecular weight of 21 600 the turnover number is 54 000.

Effect of pH *on the reaction equilibrium.* Fig. 6 shows the values of the equilibrium constant $K = [ATP][AMP]/[ADP]^2$ obtained at various conditions of pH. Each value is an average figure obtained by approaching equilibrium from both sides of the reaction. It will be seen that at low pH values, where the ionization of ADP is suppressed, the enzymic dismutation of ADP is facilitated as compared with the reverse reaction.

Properties of the sulphydryl groups of myokinase and the stability of the enzyme. It was originally reported by Colowick & Kalckar (1943) that myokinase contained SH groups, the oxidation of which resulted in the disappearance of enzymic activity. Full restoration of activity was obtained on adding reduced glutathione or cysteine. Our partially purified myokinase was entirely inactivated after dialysis for 48 hr. in cellophan tubing. Activity was not restored by addition of a concentrate of the dialysate, but the enzyme was almost entirely reactivated on addition of cysteine. Although partially purified solutions of myokinase were stable in glass vessels, the purest preparations were

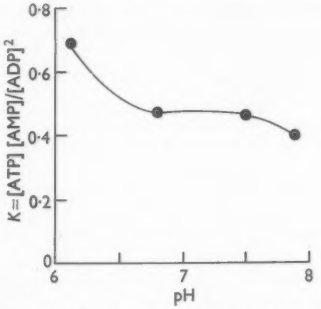

Fig. 6. Effect of pH on equilibrium of myokinase reaction (see text) after incubation for 4 hr. at 30°. At zero time, the reaction mixture (4 ml.) consisted of 0·075 M-2-amino-2-hydroxymethylpropane-1:3-diol buffer, 0·015 M-ADP or 0·0075 M-ATP + 0·0075 M-AMP, 0·01 M-$MgCl_2$, 0·006 M-cysteine hydrochloride and 1 μg. of myokinase. The reaction was stopped by adding 0·1 vol. of 30% (w/v) trichloroacetic acid.

Table 2. *Stability of myokinase solutions in various types of containers (see text)*

The solutions were stored at 5° and neutral pH.

Preparation	Concn. (mg./ml.)	Treatment: Nature	Treatment: Duration	Activity recovered (%)	Activity recovered on adding cysteine (1 mg./μg. of protein)
Purified 25-fold	0·63	Stored in glass	3 weeks	100	100
	0·22	Stored in glass	3 weeks	95	95
	0·63	Stored in glass	4 weeks	95	95
	0·63	Dialysed in cellophan	48 hr.	0	95
	0·22	Dialysed in cellophan	48 hr.	0	90
Purified 60-fold	0·69	Stored in glass	1 week	0	—
	0·69	Stored in glass	96 hr.	0	40
	0·69	Dialysed in cellophan	48 hr.	0	90
	2·7	Dialysed in cellophan	48 hr.	0	95
	2·7	Dialysed in collodion bag	48 hr.	10	80
	1·28	Dialysed in collodion bag coated with cytochrome *c*	72 hr.	100	100
	2·7	Dialysed in collodion bag coated with cytochrome *c*	48 hr.	50	90
	10·5	Dialysed in collodion bag coated with haemoglobin	48 hr.	85	100
	2·7	Dialysed in collodion bag coated with haemoglobin	48 hr.	60	90
	0·69	Stored in polythene tube	8 weeks	100	100
	0·69	Stored in Lusteroid tube	8 weeks	80	90

inactivated by contact with both glass and cellophan. In these cases, partial restoration of activity was obtained by adding cysteine, but prolonged exposure, especially to glass, resulted in irreversible inactivation (i.e. not modified by cysteine) and the appearance of a protein precipitate with fibrous strands, which imparted a flow birefringence to the solution.

Table 2 summarizes the effect of glass and cellophan on myokinase solutions, as well as the efficacy of storage in polythene and Lusteroid vessels. For kinetic experiments, polythene tubes are suitable, and the enzyme should be first activated by incubating the requisite enzyme sample at room temperature with neutral cysteine solution (1 mg. of cysteine/μg. of myokinase/ml.).

Acid and heat stability. Colowick & Kalckar (1943) established that the myokinase activity of muscle extracts was largely stable to heating at 90° for 1–2 min. in 0·05N-HCl, and that a preparation approx. four times purified retained 79% of its activity after 10 min. at 100° in 0·1N-HCl. These authors treated the reneutralized enzyme solution with reduced glutathione before assay, from which it would appear that there was inactivation after the heat–acid treatment, although no figures were given.

Table 3 shows the degree of inactivation of highly purified myokinase after heating in acid. Heating resulted in a loss of activity, but activity was recovered on subsequent addition of cysteine. If excess of cysteine was present during the heating not only was greater activity detected, but further treatment with cysteine had little effect. It appears that the first effect of heating is to cause oxidation of SH groups; the oxidized enzyme then undergoes irreversible inactivation or denaturation. The stabilizing effect of accompanying proteins on myokinase has been reported by Bowen & Kerwin (1956). On the basis of the above experiment, the present author believes that the protective action of such proteins is due to their ability to reduce the sulphydryl groups of myokinase at the expense of their own. The results given in Table 4 show the protective action of three sulphydryl-containing proteins on myokinase. The results are also consistent with the view that sulphydryl-containing proteins in the crude muscle extracts, from which myokinase is purified, are responsible for the greater stability of the enzyme in such solutions, since the purer preparations of enzyme are more prone to irreversible denaturation over long periods (see Table 2). Titration of sulphydryl groups in crude extracts (see above) indicated that there is a 40-fold excess of sulphydryl groups over those belonging to myokinase.

Effect of salts and other proteins on heat stability. Both myokinase and actomyosin were precipitated by heating in the absence of ions, the former being almost entirely irreversibly inactivated (Tables 3 and 4). Variable amounts of myokinase were recovered if HCl was present, however, and over 90%

Table 3. *Effect of heat and acid on myokinase activity, and the protective action of cysteine*

The enzyme (82 μg., highly purified) was heated in a total volume of 2 ml. at 100° for 10 min. After cooling in ice–water, and neutralizing to pH 7, portions were removed for spectrophotometric assay. All heating was carried out in polythene tubes.

Additions	Activity recovered (%)	Activity recovered (%) after treatment with cysteine
None	0	15
HCl (0·1N)	20	40
HCl (0·1N) + 2 mg. of cysteine	70	70
HCl (0·1N) + 2 mg. of cysteine	85	90

Table 4. *Protective effect of proteins on myokinase solutions*

The myokinase, highly purified, was heated in a total volume of 2 ml. at 90° for 10 min., cooled in ice–water and neutralized, pH 7. All heating was carried out in polythene tubes. The actomyosin preparation (Szent-Gyorgyi, 1951) was free of myokinase activity. Control experiments also showed that simple addition of the proteins to myokinase solutions did not interfere with the recovery of enzyme activity. All figures of recovery represent the average value of duplicate experiments.

Amount of enzyme (μg.)	Other proteins added	0·1N-HCl	0·16M-NaCl	Recovery (%)	Recovery (%) after treatment with cysteine
82	None	−	+	57·5	72·5
82	None	−	−	0	12·5
82	None	+	+	0	15
82	None	+	−	0	12·5
82	300 μg. of actomyosin	−	+	60	92·5
82	300 μg. of actomyosin	−	−	10	15
82	300 μg. of actomyosin	+	+	50	65
82	300 μg. of actomyosin	+	−	62·5	80
82	300 μg. of haemoglobin	+	−	60	80
82	300 μg. of preheated actomyosin	+	−	25	42·5
90	300 μg. of albumin (single expt.)	+	+	25	50

of the activity was recovered if NaCl was present. The failure of actomyosin and of pre-heated myosin to preserve myokinase activity under salt-free conditions was probably due to precipitation of these added proteins, or to a coprecipitation with myokinase, since it is known that the latter is strongly adsorbed by myosin.

Effect of heating in glass tubes. A comparison of the effects of heating the enzyme for 10 min. in polythene or glass tubes showed that similar recoveries of enzyme were obtained. Since glass has been shown to cause oxidation of the pure enzyme, whereas polythene does not (Table 2), it is inferred that heat itself causes oxidation and irreversible inactivation.

DISCUSSION

From the foregoing description of the properties of myokinase it is apparent that the sulphydryl groups of the enzyme determine the state of activity. Two processes are involved in the denaturation of the enzyme. The first stage involves a reversible oxidation of sulphydryl groups. In the second stage, the oxidized enzyme is irreversibly inactivated, resulting in aggregation and insolubility. Myokinase in its active form is largely stable to heating in acid. The oxidized enzyme is more easily denatured. The absence of ions appears to render the enzyme less stable, but the primary condition for denaturation appears to be oxidation. The action of glass as a denaturing agent has not been further investigated. It is possible that traces of metals in solution are responsible for oxidation, and that glass may yield such trace metals.

The protective action of added proteins appears to be due, not to physical forces such as adsorption of the enzyme by van de Waals's or ionic forces, but to their possession of a specific chemical group, the sulphydryl, which keeps in the reduced state the sulphydryl groups of myokinase. This explanation for the protective effect of added proteins on myokinase may well be found to apply to other cases of protection of enzymes by inert proteins.

Since myokinase appears to possess two sulphydryl groups/molecule and does not polymerize on oxidation, the oxidation may involve the formation of a disulphide link in each molecule.

SUMMARY

1. The preparation of myokinase, homogeneous by electrophoretic and ultracentrifugal analysis, and of constant specific activity is described.

2. Pure myokinase is very readily oxidized by exposure to glass or cellophan; such treatment predisposes the enzyme to denaturation. The reactivation of the oxidized enzyme with cysteine shows that the enzyme contains highly reactive sulphydryl groups.

3. Protection of the sulphydryl groups with cysteine, or other sulphydryl-containing proteins, endows the enzyme with considerable stability to heating and acid conditions.

4. Titration of the sulphydryl groups of myokinase indicated that the enzyme possesses two groups/molecule. A molecular weight of 21 600 is postulated.

5. Ultracentrifuge studies indicated that myokinase does not polymerize on oxidation.

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The Partial Purification of Parathyroid Hormone by Means of Ultrafiltration and Displacement Chromatography

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Although apparently the first effective parathyroid extract was prepared by Moussu in 1898, some time elapsed before Hanson (1925) and Collip (1925) clearly demonstrated that an active extract of consistent potency could be prepared by using hot dilute hydrochloric acid. Since that time there have been a number of attempts to purify such extracts further. Some further purification was achieved by Ross & Wood (1942) and L'Heureux, Tepperman & Wilhelmi (1947) by means of ammonium sulphate fractionation and fractional precipitation with acetone respectively. Several brief reports, including the one of Handler, Cohn & Dratz (1954), have appeared on the unsuccessful attempts to use chromatography for isolation of the active principle.

Because they felt that initial extraction with hydrochloric acid might be disadvantageous in later purification procedures, Davies & Gordon (1955) adopted acetic acid as the extracting agent. They showed that the active principle could be obtained by extraction with 80% acetic acid, and further demonstrated that 50–65% of the extracted hormonal activity would pass a cellophan membrane during ultrafiltration at pH 2·4. Their most potent preparations had a specific activity comparable with, or slightly greater than, those of L'Heureux *et al.* (1947).

The studies reported in this communication are concerned with an evaluation of these two acid extraction procedures and the further purification of the extracts by fractional precipitation with acetone, ultrafiltration, and displacement chromatography from ion-exchange resins. By these means a hormonally active material with a specific activity of 75–100 units/mg. dry weight has been prepared. In addition, evidence is presented which suggests that the active material obtained by means of acetic acid and hydrochloric acid extraction respectively have different chemical properties.

EXPERIMENTAL

Assay method

The increase in the concentration of calcium in the plasma of parathyroidectomized rats, 6–18 hr. after the test injection, was used as the basis of all assays. Initially an 18 hr. assay was used. This was similar to that described by Davies, Gordon & Mussett (1954), except that smaller rats (120–130 g.) were employed, and the concentration only of plasma calcium, rather than of both calcium and magnesium, was measured. Also the animals were initially used 4 days after parathyroidectomy and every 4 days thereafter for a maximum of four times and then discarded. The smaller animals were more responsive to a standard dose of Parathormone (Eli Lilly and Co.), 50 units eliciting an increase in plasma calcium of 2·2 mg./100 ml. 18 hr. after injection, compared with an increase of 1·8 mg./100 ml. after 60 units in the larger animals used by Davies *et al.* (1954). A straight-line relationship was found between the increase in plasma calcium concentration and log dose of hormone over the range of 25–125 units. Those animals whose initial calcium concentrations were 8·5 mg./100 ml. or more were not used, so that the initial plasma-calcium concentrations ranged between 6·5 and 8·4, with a mean of 7·3 mg./100 ml. Within this range it was not possible to establish any dependence of the response on the initial calcium concentration as described by Davies *et al.* (1954), even though this was evaluated in all assays performed over a 10 month period.

For screening a large number of fractions for hormonal activity 6–8 rats/fraction were used and cross-over experiments were not carried out. In this way approximate potencies were determined. When a more precise measurement of potency was desired a cross-over design similar to that of Davies *et al.* (1954) was employed. A group of 32 animals was divided into four groups; two groups received 50 and 100 units respectively of the standard (Parathormone, Eli Lilly) and the others received two different doses of the unknown, calculated as nearly as possible to contain 50 and 100 units of activity. The limits of error of this method were 75–130% when expressed as a percentage of potency.

As the purification studies progressed it was found that the time-course of action of the active material was altered (Rasmussen & Westall, 1956). The maximum response occurred at 6 rather than 18 hr. and the effect was over in 14–16 hr.

This assay method could be used, however, if the material to be tested was suspended in arachis oil rather than dissolved in 0·9% saline before injection. When the partially purified extracts were suspended in oil their time-course of action was similar to that of the crude extracts. Suspension of the crude material in oil did not change its time-course of action.

It was found more convenient to use a shorter assay. A method similar in detail to the one above was developed, except that the second bleeding was 6 rather than 18 hr. after injection. The materials to be tested were all dissolved in 0·9% saline before injection, and both the crude and more purified material could be assayed. The limits of error were found to be similar to those above. Although the maximum

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response to the standard was 15–20 hr., the response at 6 hr. to 50 units was only slightly less (1·9 compared with 2·2 mg./100 ml.) and was no more variable. When the same partially purified preparation was tested by the 6 hr. (saline) and 18 hr. (oil) methods, no difference in potency was found.

Plasma-calcium estimations

Blood (0·8–1·0 ml.) was drawn into a heparinized syringe by cardiac puncture under light ether anaesthesia. The plasma was separated as soon as possible. Calcium was determined on 0·2 ml. samples of plasma by titration at pH 12 with sodium ethylenediaminetetra-acetate (EDTA) in a Unicam SP. 600 spectrophotometer at 510 mμ, with ammonium purpurate as an indicator. The percentage transmission was recorded on semi-log graph paper after the addition of each measured increment of standard EDTA. When these readings were plotted the resulting line showed two slopes, the intersection of which was taken as the end point. This method was reported by Kenny & Toverud (1954).

Preparation of fat-free gland powder

Fresh, frozen, bovine glands were minced in a meat grinder by using a plate with $\frac{1}{4}$ in. holes. The ground tissue was placed immediately in cold acetone (0°) and reminced with a plate having $\frac{1}{16}$ in. holes. It was then extracted with acetone and chloroform as described by L'Heureux *et al.* (1947). The fresh glands (1 kg.) yielded approximately 100 g. of dry powder. The dry powder was stored in a glass-stoppered bottle at 0°, and was stable for at least 4 months. This powder was the starting material for all subsequent work.

Preparation of acetic acid extract

The procedure of Davies & Gordon (1955) was used, with some modifications. The defatted powder was dissolved in 80% (v/v) acetic acid (12 ml./g. of dry powder), heated with stirring to 75° and kept at this temperature for 5 min. The mixture was then cooled to 40°, filtered through six layers of cheese-cloth, re-extracted for 5 min. with another volume of 80% acetic acid at 75° and refiltered. The combined filtrates were cooled to 10° and 3 vol. of cold acetone (0°) was added. This mixture was allowed to stand for 1 hr. at 0°. The precipitate which formed was removed by centrifuging and discarded. Acetone was then added to the supernatant fluid to a final concentration of 90%, and the mixture allowed to stand 2–3 hr. at 0°. A precipitate formed which settled to the bottom of the flask during this time. The bulk of the clear supernatant fluid was removed by decantation and the precipitate then collected by centrifuging, washed four times with acetone and dried *in vacuo* at 0° over H_2SO_4. From 60 g. of defatted powder, approximately 3 g. of a pale-brown powder was obtained.

Preparation of hydrochloric acid extract

The procedure of L'Heureux *et al.* (1947) was used, except that the final precipitation with 90% acetone at neutrality was omitted owing to difficulties in controlling the pH. In the method used the powder was extracted with 0·2 N-HCl (10 ml./g. of powder) at 75° for 5 min. The mixture was cooled to 45° and centrifuged, and the residue re-extracted with another volume of 0·2 N-HCl at 75° for 5 min. The combined supernatant fluids were cooled to 10°. Acetone (4 vol.) was then added, and the mixture was allowed to stand 1 hr. at 0° to ensure maximal precipitation and then centrifuged to remove the precipitate. The supernatant fluid was freed from acetone by distillation under reduced pressure at a temperature not exceeding 40°. The resulting acid solution (pH 1·8) was then ultrafiltered.

Ultrafiltration

Ultrafiltration was carried out by using sacs of Visking dialysis tubing ($1\frac{1}{2}$ in. in diameter, 15 cm. long) which were reinforced with an outer sheath of open-mesh silk fabric. The construction of the sacs was demonstrated to us by Dr A. H. Gordon. Thus supported they gave adequately rapid filtration (5 ml./hr.) and could withstand a pressure of 720 mm. Hg.

For ultrafiltration, 1 g. of the powder obtained by acetone precipitation of the acetic acid extract was dissolved in 100 ml. of either 2·2 N-acetic acid (pH 2·35) or 0·2 N-formic acid (pH 2·05).

Ion-exchange columns

Both Dowex 50 (2% cross-linked) and Dowex 50 (4% cross-linked) have been used in our studies. A column containing a bed of 200–400 mesh resin 20 cm. deep and 5·5 cm. in diameter served for the fractionation of the ultrafiltrate obtained from 60 g. of the acetone-dried powder. The resin, which was used in the H^+ ion form, was allowed to stand for 1 hr. in 2 N-HCl, washed twice with water by decantation and then poured into the column as a slurry. Before use, the packed column was washed with distilled water until the pH of the effluent was almost neutral. A smaller column of the anion-exchange resin Dowex 2 (8% cross-linked) was also used. The bed of 200–400 mesh resin was 10 cm. deep and 1·5 cm. in diameter. This resin was used, according to need, either in the OH^- ion form, obtained by treatment with N-NaOH and then distilled water, or in the formate form, achieved by saturating the resin with 1·0 N-formic acid before packing and washing the column.

RESULTS

Initial extraction

The dilute hydrochloric acid and the acetic acid methods were about equally effective in extracting hormonal activity from the acetone-dried powder. A weight of 60 g. of powder yielded 10 000–12 000 U.S.P. units.

Acetone fractionation

Initially the procedure of L'Heureux *et al.* (1947) was employed to obtain an active powder from the crude hydrochloric acid extract. The crucial step in their procedure was the neutralization of the acid acetone solution, followed by the addition of more acetone to a final concentration of 90%. We were able to obtain an active powder by this means which had a potency of 20–25 units/mg. dry weight. However, great difficulty was encountered in controlling the pH during neutralization, since the protein which precipitated seemed to carry the water with it and selectively concentrate the sodium hydroxide so that the pH of the protein solution in the bottom of the flask was strongly

alkaline at a time when the supernatant was still acid. In addition, when the supernatant from the final acetone precipitation was concentrated and assayed, it was found to contain approximately 10 % of the estimated original activity. Because of these two reasons this procedure was abandoned.

Ultrafiltration

When the dilute hydrochloric acid extracts were ultrafiltered, under optimum conditions 90–95 % of the hormonal activity passed through the membrane. This value, however, was not always attained. The yield was found to be correlated with the fat content of the original dried powder, completely fat-free powders giving the highest yield. In one instance when poorly defatted powder was used the yield was approximately 50 %. When the residue in the sac was lyophilized, re-extracted with chloroform and acetone, dissolved in 0·2N-hydrochloric acid and again subjected to ultrafiltration, 90 % of the estimated activity then passed through the membrane.

Ultrafiltration of the 80 % acetic acid extract confirmed the finding of Davies & Gordon (1955) that 60–65 % of the hormonal activity passed through the cellophan membrane. Their studies were based primarily on a mouse-urine phosphate-assay method (Davies, Gordon & Mussett, 1955), but they also assessed blood-calcium-raising activity and reported a value of approximately 20 units/mg. dry wt. A similar activity was found with our material.

With the acetic acid extracts we were never able to get more than a 65 % yield in the ultrafiltrate, even from well defatted powders when 2·2M-acetic acid was used. There is no doubt that the acetic acid extracts had a larger percentage of inert material since it had a lower specific activity and was much more viscous than the hydrochloric acid extracts. It was possible to increase the yield, however, by dissolving the protein material in 0·2N-formic acid rather than in 2·2N-acetic acid. Under these conditions the fluid appeared to be less viscous and 80–90 % of the activity passed through the membrane, with a corresponding increase in specific activity of the ultrafiltrate (Table 1).

Displacement chromatography of hydrochloric acid extract

In a typical experiment the extraction of 60 g. of dried powder with 0·2N-hydrochloric acid yielded 600–700 ml. of ultrafiltrate. This volume was doubled by the addition of water to reduce the acid concentration. This solution was allowed to run on to a Dowex 50 resin column at a rate not exceeding 3 ml./min. A definite, but only faintly visible, band was seen to form and to progress slowly down the column. The front of this band extended to a point about two-thirds of the way down the column when all the diluted ultrafiltrate had been applied. The column was then washed with 0·01N-hydrochloric acid until the effluent no longer absorbed light at 225 mμ (measured in a Unicam SP. 500 spectrophotometer).

Displacement of the substance held by the resin was then carried out at a flow rate of about 30 ml./hr. with a solution obtained by mixing equal volumes of 0·2N-sodium hydroxide and 0·02N-hydrochloric acid. As displacement proceeded a band of white insoluble material (later tentatively identified as guanine) formed just ahead of the sodium front (dark band). This precipitated material tended to distort the sodium front, but did not interfere unduly with development provided that the recommended flow rate was not exceeded. The faint protein band did not move forward until the sodium front approached within 6–8 cm. of it.

Collection of the effluent in 10–20 ml. fractions began as soon as increased absorption at 225 mμ was observed, and continued until there was a sharp rise in absorption at 260 mμ indicating breakthrough of the purine front (Fig. 1). The fractions which contained protein, but not purine, were pooled and passed through a column of Dowex 2 resin in the formate form and then lyophilized. The exchange of formic for hydrochloric acid permitted a more

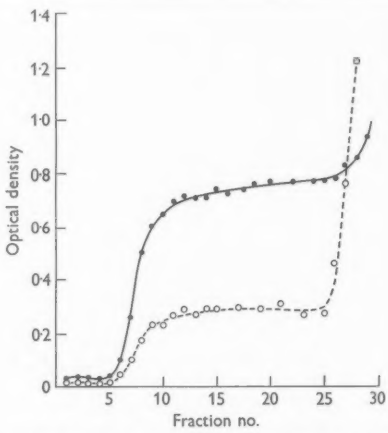

Fig. 1. Ultraviolet absorption at 225 mμ (●) and 260 mμ (○) of fractions obtained by displacement chromatography of parathyroid hormone on Dowex 50 (4 % cross-linked) (200–400 mesh). The material applied to the column was an ultrafiltrate of hydrochloric acid extract of dried glands. The developing solution was 0·09M-NaOH–0·01M-NaCl. Fractions were 15 ml. each, and the hormonal activity was found in fractions 6–25. The sharp rise in absorption at 260 mμ beginning in fraction 26 indicates the breakthrough of the purine band.

satisfactory lyophilization. The powder from the lyophilized fractions was found to have an activity of from 75 to 100 units/mg. dry wt. Some further hormonal activity was always found in the effluent obtained during the development of the purine band due to the previously mentioned irregularity in this front. This material could be recovered by passing it through a Dowex 2 column in the OH^- ion form to remove the purine, reacidifying and rerunning it on a smaller Dowex 50 column.

From 60 g. of the original fat-free gland powder 90–100 mg. of this highly active material was obtained. It has been stored for as long as 6 months, as a lyophilized powder in a vacuum desiccator at room temperature, without change in potency.

Displacement chromatography of acetic acid extracts

The powder obtained by lyophilization of the ultrafiltrate of the acetic acid extract (obtained from the same fat-free gland powder as the hydrochloric acid extract) was taken up in 0·1 N-hydrochloric acid and applied to a Dowex 50 (4% cross-linked) column in the H^+ ion form. However, with these extracts no visible band formed on the column and it was established that the active material was not retained by the resin but passed straight through the column, so that purification was not achieved. Heating this powder to 75° for 5 min. in 0·2 N-hydrochloric acid did not change its chromatographic behaviour. Likewise, heating the hydrochloric acid ultrafiltrates in 80% acetic acid for 5 min. at 75° did not alter its chromatographic behaviour. This would seem to indicate a difference between the active molecules obtained by means of the two acid-extraction procedures, and an inability to convert one into the other, at least in the circumstances described.

Specific activity

Table 1 summarizes the data on the degree of purification achieved by each of the procedures employed, as well as the average recovery of originally extracted activity. The potency of the original acetic acid extract was approximately one-half that of the hydrochloric acid extract. Both were purified to approximately the same degree by acetone fractionation and ultrafiltration, but only the hydrochloric acid extract was further purified by displacement chromatography on Dowex 50 resins. This highly active material (75–100 units/mg. dry wt.) had a nitrogen content of 14·0% (micro-Kjeldahl), which indicated a specific activity of 500–700 units/mg. of N.

When the active precipitate obtained by acetone fractionation of the acetic acid extract was dissolved in 0·2 N-formic acid (Table 1*b*) rather than 2·2 N-acetic acid (Table 1*a*) a greater percentage of the activity passed through the cellophan and the specific activity of the ultrafiltrate was correspondingly higher (30–35 rather than 15–20 units/mg. dry wt.) and the overall recovery was consequently greater (80–90% as compared with 50–60%).

Electrophoresis and ultracentrifugation

Preliminary studies have been carried out on our most potent hydrochloric acid-extracted preparation (100 units/mg. dry wt.). Electrophoresis in a Tiselius apparatus was performed by K. Schmid (Massachusetts General Hospital, Boston, Mass.), using a glycine buffer, pH 2·5. The pattern obtained showed that the material was heterogeneous, consisting of two and possible three components. Ultracentrifuging performed by A. H. Gordon and P. A. Charlwood (National Institute for Medical Research, London, N.W. 7) showed that the material behaved as if it consisted primarily of a single component with an estimated molecular weight of 4000–6000.

Other constituents of parathyroid glands

During the course of this work, we have gained some knowledge about the chemical constituents of parathyroid-gland tissue. Apart from the tissue

Table 1. *Purification of parathyroid hormone*

Specific activities after each purification procedure, and the percentage of the activity in the original extracts recovered after each step are given for material extracted initially with either 80% acetic acid or 0·2 N-HCl (see text). In the 80% acetic acid columns (*a*) and (*b*) refer to the specific activities and yields obtained with 2·2 N-acetic and 0·2 N-formic acid respectively, during the ultrafiltration step.

Stage of preparation	Hormonal activity (units/mg. dry wt.)		Recovery (%)	
	80% acetic acid	0·2 N-HCl	80% acetic acid	0·2 N-HCl
Original extract	3–5	8–10	100	100
Supernatant from 75% acid, acetone fractionation	—	12–15	—	100
Precipitate from 90% acetone fractionation	7–10	20–25	90–95	80
Ultrafiltrate	(*a*) 15–20 (*b*) 30–35	30–40	(*a*) 50–60 (*b*) 80–90	90–95
Effluent after displacement chromatography on Dowex 50 (4% cross-linked)	—	75–100	—	75–80

protein which is removed by acetone precipitation and ultrafiltration there is a considerable amount of purine tentatively identified as guanine by means of paper chromatography, paper electrophoresis, and a positive Pauly reaction. The effluent from the Dowex 50 column contains at least two recognizable substances: one has been identified as inositol, m.p. 224° [mixed m.p. with *meso*inositol (m.p. 225°), 224°] (Found: C, 39·7; H, 6·5. Calc. for $C_6H_{12}O_6$: C, 40·0; H, 6·7 %). The other is a peptide (shown by liberation of amino acids on acid hydrolysis) which is held by and can be displaced from a Dowex 2 column. In addition there are free amino acids.

DISCUSSION

Because the initial electrophoresis experiments showed that the most active material was heterogeneous, further characterization was not done. From these data and the results of the ultracentrifuge experiment, it would appear that our most active preparation, obtained from the hydrochloric acid extract by means of ultrafiltration and displacement chromatography on Dowex 50 resin, is a mixture of polypeptides of approximately the same size, in a molecular-weight range of 4000–6000. This is similar to the findings of Davies & Gordon (1955) with acetic acid extracts of lower hormonal potency. Our active material has a potency of 500–700 units/mg. of nitrogen. This is approximately twice the potency found by L'Heureux *et al.* (1947) in material prepared by acetone fractionation. The basis of their assay method was the fall in serum inorganic phosphorus concentration of normal rats (size not stated) 3 hr. after the injection of the test material (Tepperman, L'Heureux & Wilhelmi, 1947). Because of the difference in assay method it is difficult to make a strict comparison. However, an extract prepared according to their method was found to have an activity of approximately 25 units/mg. dry wt. by our assay method. The material we obtained by displacement chromatography is the most active yet reported, except for that described by Raoul, Marnay & Prelot (1955). Upon repeating their work with a more reliable assay method it has not been possible to confirm their findings (Rasmussen & Westall, 1957). Actually, as reported, no hormonal activity could be demonstrated in their so-called 'lipoid fractions', and 80 % of the estimated original activity was found in the protein residues, results completely contradictory to theirs. Because Raoul *et al.* (1955) used rabbits as assay animals it is difficult to accept their findings, particularly since Dyer (1935), who developed the method, concluded that it was unsuitable for quantitative assays. Certainly the majority of past evidence [summarized by Greep (1948) and Greep & Kenny (1955)], as well as the recent work of Davies & Gordon (1955) and our own experience, indicate that this hormone is a protein.

Our experience with ultrafiltration confirms and extends the work of Davies & Gordon (1955). To obtain maximal yield during the procedure, it is necessary that the initial fat extraction be thorough. This does not wholly explain the difference in yield obtained with the two different acid extracts. The greater viscosity of the acetic acid extracts may, in part, explain the lower yield. The improvement of yield with 0·2 N-formic acid suggests that pH may be important in controlling the degree of dissociation between the active hormone and larger proteins. This might also explain why high yields are obtained with hydrochloric acid extracts. Alternatively, it may be that the hormone exists as a larger complex in the natural state and is more thoroughly cleaved into smaller, but still active, units by the hydrochloric acid.

Although by using the chromatographic conditions described we were able to carry out successful displacement development, the performance was not ideal. This was due primarily to the precipitation of purine in front of the sodium band which interferes with displacement development. As pointed out, this difficulty was minimized if the recommended flow rate was not exceeded. Apparently the sequence of events on the column is as follows: the purine is soluble in the acid solution in which it is applied to the column; when displacement development is begun, the sodium in the developing solution displaces the purine from the column, but because the conditions at this point are close to neutrality the purine precipitates; then as the resin is saturated with sodium the pH rapidly rises to 12–13 and the purine redissolves. So far we have been unable to circumvent this difficulty, but it may be possible to separate the purine from the protein before displacement chromatography by use of a dialysis cell similar to that described by Craig & King (1955). The use of dilute chloride (0·01 N) in the developing solution was necessary to provide acidic conditions in the ambient solution ahead of the sodium front in order to prevent precipitation of protein on the resin. If the sodium hydroxide was replaced by either ammonium hydroxide (0·1 N) or glycine (0·1 N) in the developing solution, displacement of the protein band did not occur; instead the protein remained on the resin and was overrun by the base. Because the rate of equilibration of the protein with the resin is quite slow it is imperative to apply the protein solution slowly. Even with the rate of application recommended it is obvious that complete saturation of the resin did not occur, since the protein front did not begin to move until the sodium front had progressed approximately one-third the way down the column. The reason for part of this behaviour is no doubt that the purine which

precipitates no longer takes up its space on the resin.

Because of the difference in behaviour of the acetic acid and hydrochloric acid extracts when subjected to the same chromatographic procedure it seems safe to conclude that they differ chemically. This is further borne out by the difference in their stability in various buffer systems, and their electrophoretic properties (Dr H. Rasmussen, unpublished work). For sake of reference, we have called the active material extracted by hydrochloric acid, Parathormone A (PTH-A) and that extracted by acetic acid, Parathormone B (PTH-B).

SUMMARY

1. A method is described for the partial purification of parathyroid hormone by means of hydrochloric acid extraction, acetone fractionation, ultrafiltration and displacement chromatography on ion-exchange resin.

2. The procedure yields material of potency 75–100 units/mg. dry weight (500–700 units/mg. of N) in quantities of 90–100 mg./600 g. of fresh bovine glands.

3. This material is heterogeneous on electrophoresis at pH 2·5.

4. Evidence is presented which suggests that the active material obtained by means of acetic acid and hydrochloric acid extraction respectively have different chemical properties.

We wish to thank Dr A. H. Gordon for his constant interest and many helpful suggestions. Our thanks also to Professor C. E. Dent and Professor M. Rosenheim for their support of, and interest in, this work, which was initiated at their suggestion. This work was supported in part by grant A-1331 of the U.S. Public Health Service, and was carried out while one of us (H.R.) was a recipient of a U.S. Public Health Research Fellowship.

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The Structure of Serratamic Acid

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In a previous communication (Cartwright, 1955) the isolation of serratamic acid, a metabolic product of organisms of the *Serratia* group, was described. On the basis of the nature of its hydrolytic products and lack of ninhydrin reaction a peptide structure was advanced and this has now been confirmed by synthesis. In particular, L-serine was rigidly established as a product of acidic hydrolysis. Both alkaline and acidic hydrolysis yielded an acid, $C_{10}H_{20}O_3$, which was tentatively equated with a hydroxydecanoic acid, and has now been identified as D-3-hydroxydecanoic acid.

Molecular formula of serratamic acid

Earlier experiments favoured a molecular formula $C_{13}H_{25}O_5N$ for serratamic acid but did not entirely exclude the possibility of its being a larger molecule. Subsequently serratamic acid has been converted

into serratamic acid methyl ester, $C_{14}H_{27}O_5N$, by the action of diazomethane on an ethereal suspension of the acid or by the action of methyl iodide on a methanolic suspension of the silver salt of the acid. The stability of this ester in molten camphor has enabled molecular-weight determinations to be done by the Rast method, when values in accordance with $C_{14}H_{27}O_5N$ were consistently obtained. This evidence, together with the previously reported equivalent weight, and the failure of further attempts to isolate other fragments from hydrolysates is regarded as proof that the serratamic acid molecule is represented by $C_{13}H_{25}O_5N$.

Identification of the C_{10} *acid*

Doubts about the structure of the carbon chain of the C_{10} degradation acid (Cartwright, 1955) were resolved in two ways. Branched-chain structures were eliminated by *C*-methyl determinations, which gave negative results. The presence of a *n*-C_{10} chain was confirmed by treatment of the acid with phosphoryl chloride or phosphorus pentoxide in benzene followed by catalytic reduction of the unsaturated or chlorinated product, or both; decanoic acid was thus obtained and its identity established by preparation and analysis of the anilide and *p*-bromophenacyl ester, which were also compared with the corresponding derivatives of authentic decanoic acid.

When larger quantities of serratamic acid were available a more rigorous purification of the C_{10} degradation acid was made. After vacuum distillation the acid was crystallized at low temperature from light petroleum, forming felted needles, m.p. 47°. A direct oxygen determination confirmed the formula $C_{10}H_{20}O_3$ previously allotted. Small quantities of impurity caused the substance to separate from solvents in a form only filterable with difficulty. The pure substance showed minor changes in infrared spectrum (Fig. 1) and the resemblance of the absorption to that of DL-3-hydroxydecanoic acid (Fig. 2) was more pronounced, the minor differences

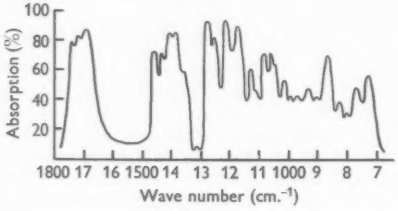


Fig. 1

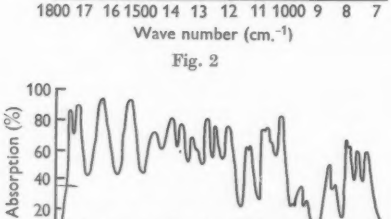


Fig. 2

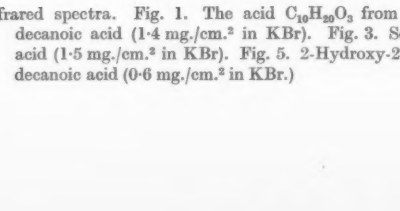


Fig. 3

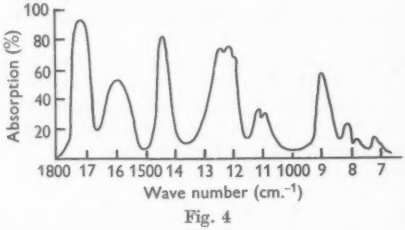


Fig. 4

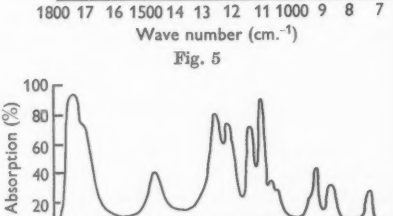


Fig. 5

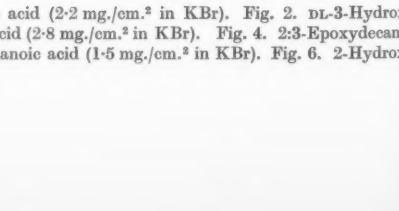


Fig. 6

Infrared spectra. Fig. 1. The acid $C_{10}H_{20}O_3$ from serratamic acid (2·2 mg./cm.² in KBr). Fig. 2. DL-3-Hydroxydecanoic acid (1·4 mg./cm.² in KBr). Fig. 3. Serratamic acid (2·8 mg./cm.² in KBr). Fig. 4. 2:3-Epoxydecanoic acid (1·5 mg./cm.² in KBr). Fig. 5. 2-Hydroxy-2-methylnonanoic acid (1·5 mg./cm.² in KBr). Fig. 6. 2-Hydroxydecanoic acid (0·6 mg./cm.² in KBr.)

being no greater than might reasonably be attributed to optical configuration. Accordingly, the optical properties of the acid were re-examined. No measurable rotation was observed in ethanol solution, but in chloroform activity was demonstrated, the acid having $[\alpha]_D^{20} - 19 \pm 1°$ (*c*, 2·1). By a tedious fractional crystallization of the cinchonidine salts of DL-3-hydroxydecanoic acid (the configurations of which are deduced from evidence presented below) the enantiomorphic forms were obtained. The less soluble cinchonidine salt was that of D-3-hydroxydecanoic acid, having m.p. 119°, $[\alpha]_D^{20} - 82{\cdot}5 \pm 0{\cdot}5°$ in chloroform (*c*, 2·0), yielding an acid having m.p. 47°, $[\alpha]_D^{20} - 17{\cdot}5 \pm 2°$ in chloroform (*c*, 0·9). The more soluble cinchonidine salt was that of L-3-hydroxydecanoic acid, m.p. 98°, $[\alpha]_D^{20} - 67 \pm 0{\cdot}5°$ in chloroform (*c*, 1·27). It gave an acid, m.p. 46°, $[\alpha]_D^{20} + 18 \pm 2°$ in chloroform (*c*, 0·9). A mixture of equal parts of the antipodes melted at 57°. Admixture of the degradation acid with the laevorotatory form gave no depression in melting point, whereas admixture with an equal weight of the dextrorotatory form gave m.p. 57°. The latter mixture did not depress the melting point of DL-3-hydroxydecanoic acid. The antipodes of 3-hydroxydecanoic acid are more soluble in petroleum than is the racemic acid. With the small quantities available the rotation of the antipodes in ethanol solution did not exceed the possible experimental error of $\pm 2°$.

Subsequent to this study Serck-Hanssen & Stenhagen (1955) and Serck-Hanssen (1956) published a synthesis of L-3-hydroxydecanoic acid having $[\alpha]_D^{22} + 20°$ in chloroform and $-3°$ in ethanol, starting from precursors of known absolute configuration. This substance was shown to be the antipode of an acid isolated from natural sources by Bergström, Theorell & Davide (1946).

Through the courtesy of Dr Serck-Hanssen, observations were made on the mixed-melting-point behaviour of his acid with the antipodes from DL-3-hydroxydecanoic acid and the acid obtained from serratamic acid. Both laevorotatory 3-hydroxydecanoic acid and the degradation acid, when mixed separately with equal weights of Serck-Hanssen's acid, showed m.p. 57°, the mixture not depressing the melting point of authentic DL-3-hydroxydecanoic acid. There was also no depression in mixtures of the resolved dextrorotatory substance and Serck-Hanssen's dextrorotatory form. These observations, supported by a comparison of infrared spectra, including that of L-3-hydroxydecanoic acid, a copy of which was supplied by Dr Serck-Hanssen, show that the hydrolytic product $C_{10}H_{20}O_3$ is D-3-hydroxydecanoic acid and that serratamic acid provides another example of a naturally occurring derivative of this acid.

The *p*-bromophenacyl ester of D-3-hydroxydecanoic acid (from the resolved acid), m.p. 105°, exhibited no depression in melting point on admixture with the previously reported derivative of the degradation acid, and they were identical in solubility and crystal habit. It follows that the methyl ester obtained from treatment with methanolic hydrogen chloride of serratamic acid is methyl D-3-hydroxydecanoate (m.p. 13°, $[\alpha]_D^{20} - 18{\cdot}3 \pm 1°$ in chloroform *c*, 2·75). The presence of the hydroxyl group was chemically confirmed by conversion of the ester into the liquid methyl D-3-acetoxydecanoate, b.p. 73°/0·07 mm., by the action of cold acetic anhydride. The methyl ester showed no tendency to cyclic-ester formation (see below). Attempts to prepare substituted *O*-benzoyl derivatives gave either unchanged material or unsaturated compounds, the latter being also found in some experiments with the free acid. From aqueous acid hydrolysis of serratamic acid and the action of mineral acids on, and attempts at acylation on, the C_{10} acid there was always obtained a neutral oily fraction which by alkaline hydrolysis gave almost theoretical yields of the C_{10} acid.

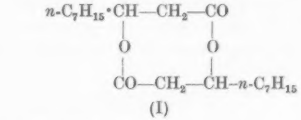
On the basis of boiling point, analytical figures and hydrolysis it must be concluded that this lactone-like substance is the cyclic ester, $C_{20}H_{36}O_4$ (I).

Structure and synthesis of serratamic acid

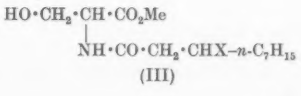
The observations of this and the earlier communication lead to the conclusion that serratamic acid is *N*-(D-3-hydroxydecanoyl)-L-serine

(II; $R^1 = R^2 = H$).

and in support of this its infrared spectrum (Fig. 3) shows absorption at 1649 and 1632 cm.$^{-1}$, attributable to the =CO of a secondary amide, and at 1549 cm.$^{-1}$, attributable to the =NH of a secondary amide.



(I)



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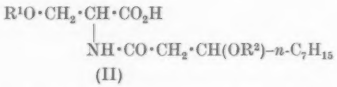
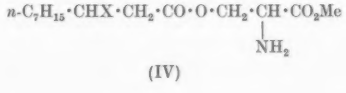


(II)



(IV)

The possible synthetic routes to serratamic acid were limited by observations of the difficulty in isolating pure derivatives of the general type (II) (R^1 and R^2 = acyl, alkyl, arylalkyl) from serratamic acid itself and the problems which could arise from the lability of the molecule in attempts to regenerate serratamic acid from such derivatives. In the direct condensation of serine and an appropriate acid chloride both *O*- and *N*-acylation can occur, and in order to limit *O*-acetylation by the desired acid chloride *O*-acetyl-DL-serine (Sakami & Toennies, 1942) was prepared for model experiments. However, the insolubility of this substance in solvents inert to acid chlorides prevented the condensation. All attempts to esterify *O*-acetylserine or to acetylate serine methyl ester to produce a more soluble derivative failed, or resulted respectively in deacetylation or demethylation. Recourse was then made to the difference in properties of products of the type (III) and (IV) (X = halogen or acyloxy) which could arise by simultaneous *O*- and *N*-acylation of serine methyl ester by the appropriate acid chloride, the desired *N*-acylated product (III) being insoluble in both dilute acid and alkali.

Because of the difficulty in preparing quantities of the synthetic optical isomers of the amino acid and hydroxy acid moieties of serratamic acid, model experiments were first carried out on the readily available DL-serine and DL-3-hydroxydecanoic acid. The action of thionyl chloride on DL-3-hydroxydecanoic acid gave a predominance of chlorine-free product consistent with the cyclic ester (I). Although pure DL-3-acetoxydecanoic acid could not be prepared by the action of acetyl chloride and pyridine on the acid in the cold, the subsequent treatment of the crude product with thionyl chloride showed that some appreciable acetylation and formation of acid chloride had occurred because condensation of this second crude product with serine methyl ester gave a neutral nitrogenous fraction. By the action of cold dilute alkali this fraction lost acetyl and methyl ester groups and gave good yields of racemic serratamic acid or DL-*N*-(3-hydroxydecanoyl)serine, m.p. 144°.

By a similar condensation serratamic acid was synthesized by recombination of the L-serine and D-3-hydroxydecanoic acid moieties obtained directly from serratamic acid itself and by condensation of commercial L-serine and D-3-hydroxydecanoic acid obtained by resolution of DL-3-hydroxydecanoic acid in this Laboratory. The two synthetic products and natural serratamic acid had identical single and mixed melting points and were identical in solubility, crystal habit and optical properties.

There was left after distillation of the crude neutral fraction from treatment of serratamic acid with methanolic hydrogen chloride a residue which solidified and which on purification formed a neutral substance, m.p. 94°, containing chlorine and methoxyl. Elementary analysis showed this to be $C_{14}H_{26}O_4Cl$, i.e. chloroserratamic acid methyl ester (V; R = Cl).

$$\begin{array}{l} R\cdot CH_2\cdot CH\cdot CO_2Me \\ \quad\quad\quad\quad | \\ \quad\quad\quad NH\cdot CH_2\cdot CH(OH)\text{–}n\text{-}C_7H_{15} \end{array}$$
(V)

The structure of this product was deduced by catalytic reduction, when the chlorine atom was removed to give deoxyserratamic acid methyl ester, m.p. 58° (V; R = H), which on hydrolysis with hydrochloric acid gave rise to α-alanine, identified by paper chromatography, showing that the hydroxyl group of the serine moiety of serratamic acid had been replaced by chlorine. Attempts to prepare larger quantities of the chloro derivative by the action of thionyl chloride and phosphorus chlorides on serratamic acid methyl ester were accompanied by extensive decomposition. The action of hydrogen chloride in different solvents and conditions gave either unchanged material or L-serine and D-3-hydroxydecanoic acid.

Properties of some oxygenated decanoic acids

Owing to apparently confusing evidence obtained in earlier stages of this study, other oxygenated decanoic acids were prepared for comparison with the C_{10} degradation acid.

2:3-Epoxydecanoic acid (infrared spectrum, Fig. 4) was synthesized by a Claisen–Darzen-type condensation between octyl aldehyde and ethyl chloroacetate. This substance is capable of existence in *cis* and *trans* forms, but an attempt to prepare a geometrical isomer by oxygen-ring fission gave a chlorohydroxydecanoic acid, m.p. 93°, but only the original epoxy acid was regenerated on dehydrochlorination with ethanolic potassium hydroxide. Suitable conditions for the reduction of the chlorine atom in the molecule could not be found and hence the position of the substituents was not established. The action of per-acids on 2:3-decenoic acid failed to yield an epoxy acid.

2-Hydroxy-2-methylnonanoic acid (infrared spectrum, Fig. 5) was synthesized by a method similar to that of Maehlmann (1915). In contrast to the degradation acid this substance was readily converted into a *p*-nitrobenzoyl derivative, m.p. 137°.

2-Hydroxydecanoic acid was also prepared and its infrared spectrum (Fig. 6) determined.

EXPERIMENTAL

All melting points are uncorrected.

Serratamic acid methyl ester. This was prepared in two ways: (*a*) A solution of serratamic acid (2 g.) in methanol (5 ml.) was added to a solution of $AgNO_3$ (1·24 g.) in

methanol (50 ml.). After the addition of K_2CO_3 (0·5 g.) in the minimum quantity of water the suspension of silver salt was refluxed with a large excess of methyl iodide for 18 hr. The solid was separated by filtration and the filtrate evaporated under reduced pressure, leaving an oil which solidified. After washing with saturated $NaHCO_3$ solution and water to remove unchanged acid, the product was dried and crystallized from amyl ether from which 1·5 g. of felted needles separated, m.p. 95° (Found: C, 58·7; H, 9·2; N, 4·6. $C_{14}H_{27}O_5N$ requires C, 58·2; H, 9·0; N, 4·8%).

(*b*) A suspension of serratamic acid (2 g.) in dry ether (100 ml.) was treated in portions with a solution of diazomethane in dry ether until an excess of methylating agent was present, as judged by the yellow colour persisting for 5 min. The filtered solution was then evaporated and the product crystallized from amyl ether. This substance, m.p. 94–95°, gave no depression in m.p. with that prepared as above.

Of several mixtures used in the Rast determination of mol.wt. the following is an example. The ester (0·0514 g.) was dissolved in camphor (0·5013 g.). The original camphor had m.p. 177·5°, and the mixture, m.p. 163·6°, 164°, 164°. The depression of 14° corresponds to mol.wt. 291. $C_{14}H_{27}O_5N$ requires 289.

Dehydration experiments on the degradation acid ($C_{10}H_{20}O_3$). (*a*) With phosphorus pentoxide. The acid (1·0 g.), P_2O_5 (2 g.) and dry benzene (25 ml.) were heated under reflux for 30 min. and, after cooling, water was cautiously added and the organic layer separated. An acidic fraction was removed from the benzene solution by extraction into saturated $NaHCO_3$ solution, from which it was isolated as an oil (0·3 g.) by acidification and extraction with ether followed by drying and removal of the solvent. This unsaturated substance was dissolved in methanol (5 ml.) and shaken with Pd–charcoal catalyst (10%, w/w; 0·05 g.) in H_2 at room temp. and pressure until the rapid absorption of H_2 ceased; the catalyst was then removed and the solvent evaporated under reduced pressure, leaving an oil which was solid at low temperature. A small quantity was pressed on a porous tile to remove a trace of oil and gave a solid, m.p. 30–31°, undepressed on admixture with decanoic acid, m.p. 31°. The bulk of the product was converted into the sodium salt by titration and evaporation to dryness, and this in turn was treated under reflux for 1 hr. with an equivalent of *p*-bromophenacyl bromide in ethanol (10 ml.). On cooling and dilution with water the *p*-bromophenacyl ester separated and was crystallized from light petroleum, b.p. 40–60°. This ester (0·4 g.), m.p. 64–65°, produced no depression of m.p. on admixture with an authentic sample of decanoic acid *p*-bromophenacyl ester, m.p. 65°.

(*b*) With phosphoryl chloride. The degradation acid (1·0 g.), $POCl_3$ (0·5 g.) and benzene (20 ml.) were heated under reflux for 2 hr. and the mixture stripped at the water pump at 100°. The residue was dissolved in ether and acidic matter separated by shaking with $NaHCO_3$ solution. Acidification of the alkaline extract precipitated oil (0·3 g.) which was isolated by ether extraction. This product, which was unsaturated (Br_2 in $CHCl_3$ was decolorized) and also contained chlorine, was dissolved in methanol (10 ml.) containing NaOH (10 drops of 2N-) and shaken with Pd–charcoal catalyst (10%, w/w; 0·05 g.) in H_2 at room temp. and pressure. When H_2 uptake ceased (40 ml. absorbed) the catalyst was removed and the solvent evaporated, leaving a residue smelling of decanoic acid. To this was added an excess of $SOCl_2$ (1·0 ml.) and the mixture heated until evolution of HCl ceased, when excess of reagent was removed *in vacuo* at 100°. The crude acid chloride was dissolved in dry ether, and aniline (1·0 ml.) was added and the mixture allowed to stand overnight, after which dilute HCl was added to remove excess of aniline from the ether phase, which was subsequently dried over anhydrous Na_2SO_4 and evaporated. The solid anilide separated from light petroleum (b.p 40–60°) in needles (0·2 g.), m.p. 64–65° (Found: C, 78·1; H, 10·0; N, 5·2. Calc. for $C_{16}H_{25}ON$: C, 77·8; H, 10·1; N, 5·7%). This substance did not depress the m.p. of decanoic acid anilide, m.p. 66°. In the two experiments above, the neutral substance remaining in the original benzene layers was treated in the same way as the similar substance obtained by the action of hydrochloric acid on serratamic acid (see below) and shown to be identical with it.

Purification of D-3-*hydroxydecanoic acid.* Serratamic acid was hydrolysed by treatment with methanolic HCl as described by Cartwright (1955). The neutral ether-soluble product was fractionated at very low pressure, the bath being carefully maintained at the fixed temperature necessary to produce slow distillation. After three distillations *methyl* D-3-*hydroxydecanoate*, m.p. 13°, was obtained (Found: C, 65·3; H, 10·8. $C_{11}H_{22}O_3$ requires C, 65·3; H, 10·9%). Equal weights of the ester, acetic anhydride and fused sodium acetate were mixed and allowed to stand 18 hr. at room temp. Excess of acetic anhydride was removed at 40° *in vacuo* and the product extracted in ether from the residue. Distillation gave 75% yields of *methyl* D-3-*acetoxydecanoate*, b.p. 72°/0·07 mm. (Found: C, 63·5; H, 9·7. $C_{13}H_{24}O_4$ requires C, 63·8; H, 9·8%).

Pure methyl D-3-hydroxydecanoate was hydrolysed in accordance with directions given by Cartwright (1955) for the less pure substance. The acidic product, D-3-*hydroxydecanoic acid*, was distilled twice in high vacuum, b.p. 115°/0·002 mm., m.p. 45–47°, and crystallized at 0° from light petroleum (b.p. 40°) in needles, m.p. 47°, $[\alpha]_D^{20} - 19 \pm 1°$ in $CHCl_3$ (*c*, 2·1) (Found: C, 63·8; H, 10·8; O (direct) 25·5. $C_{10}H_{20}O_3$ requires C, 63·8; H, 10·7; O, 25·5%).

The *cinchonidine salt* was prepared by dissolving the acid (1·27 g.) and cinchonidine (2·0 g.) in boiling acetone (50 ml.). On concentration to 25 ml. and cooling, the derivative (3·0 g.) separated as sheaves of needles, m.p. 119–120°, $[\alpha]_D^{20} - 82·7 \pm 0·5°$ in $CHCl_3$ (*c*, 2·5). The rotation of this substance was unchanged by repeated crystallization from acetone, ethyl acetate or CCl_4 (Found: C, 72·4; H, 8·8; N, 5·7. $C_{29}H_{42}O_4N_2$ requires C, 72·2; H, 8·8; N, 5·8%).

Resolution of DL-3-*hydroxydecanoic acid.* DL-3-Hydroxydecanoic acid (1·0 g.) and cinchonidine (1·5 g.) were dissolved in boiling CCl_4 (50 ml.) and filtered to remove a small amount of undissolved substance. Concentration to 25 ml. and cooling produced a crop of crystals (*A*, 1·5 g.), m.p. 90–100°. This obvious mixture was recrystallized six times from CCl_4 and finally twice from ethyl acetate to give the *cinchonidine salt* of D-3-*hydroxydecanoic acid* (0·6 g.), m.p. 119–120°, $[\alpha]_D^{20} - 82·5 \pm 0·5°$ in $CHCl_3$ (*c*, 2·0) (Found: C, 72·0; H, 9·0; N, 5·8. $C_{29}H_{42}O_4N_2$ requires C, 72·2; H, 8·8; N, 5·8%). The mother liquors from crystals *A* were gradually concentrated to produce five crops of impure crystals (which were combined with the mother liquors from the recrystallizations of substance *A* in the normal manner of fractional crystallization) to leave a gummy substance (0·7 g.) from which crystals were not readily obtained.

Liberation of the free acid from this crude salt and reconversion into the cinchonidine derivative in CCl_4 gave a small quantity of relatively insoluble material which was discarded. Addition of light petroleum (b.p. 40–60°) to the hot mother liquors gave a substance, m.p. 95–97°, which after recrystallization from the same solvents gave the pure *cinchonidine salt* of L-3-*hydroxydecanoic acid* (0·5 g.), m.p. 98°, $[\alpha]_D^{20} - 67 \pm 1°$ in $CHCl_3$ (*c*, 1·3) (Found: C, 72·0; H, 8·9; N, 5·6%. $C_{29}H_{42}O_4N_2$ requires C, 72·2; H, 8·8; N, 5·8%). Prolonged manipulation of the fractions in the centre of the fractional crystallization diagram failed to yield any homogeneous crystals of either enantiomorph. Failure was likewise recorded with alternative solvents.

The optically active acids were recovered from the purified cinchonidine salts of m.p. 120° and 98° by suspension in ether and addition of dil. HCl. The ether solutions were washed with dil. HCl and water, dried over Na_2SO_4 and evaporated to give almost theoretical yields respectively of D-3-*hydroxydecanoic acid*, m.p. 46–47° (needles from light petroleum, b.p. 40°), $[\alpha]_D^{20} - 17{\cdot}5 \pm 2°$ in $CHCl_3$ (*c*, 1·0) (Found: C, 64·0; H, 10·8. $C_{10}H_{20}O_3$ requires C, 63·9; H, 10·6%) (admixture with acid $C_{10}H_{20}O_3$ from serratamic acid gave no depression of m.p.) and L-3-*hydroxydecanoic acid*, m.p. 45–47° (needles from light petroleum, b.p. 40°), $[\alpha]_D^{20} + 18 \pm 2°$ in $CHCl_3$ (*c*, 1·0) (Found: C, 63·9; H, 10·8. $C_{10}H_{20}O_3$ requires C, 63·9; H, 10·6%). Admixture of this acid with a sample from Dr Serck-Hanssen produced no change in m.p., whereas admixture with an equal weight of the acid $C_{10}H_{20}O_3$ from the degradation of serratamic acid raised the m.p. to 57°, which was unchanged by the addition of DL-3-hydroxydecanoic acid.

The *p-bromophenacyl ester of* D-3-*hydroxydecanoic acid* was prepared by standard procedures and it formed plates or needles, m.p. 105–106° from light petroleum (b.p. 60–80°) (Found: C, 56·2; H, 6·6; Br, 20·5. $C_{18}H_{25}O_4Br$ requires C, 56·1; H, 6·5; Br, 20·8%). No depression of m.p. was observed on admixture with the corresponding derivative of the acid $C_{10}H_{20}O_3$ from the degradation of serratamic acid.

Action of hydrochloric acid on serratamic acid. Serratamic acid (5 g.) and conc. HCl (50 ml.) were heated on the water bath for 3 hr. and then cooled and diluted with water and the oil extracted into ether. Acidic material (0·2 g.) was removed from the ether layer by extraction with saturated $NaHCO_3$ solution, after which the ether solution was dried over Na_2SO_4, the solvent removed and the product distilled. After a small forerun of unsaturated material the cyclic ester (I) distilled (3 g.) with slight decomposition, b.p. 130°/0·001 mm. (Found: C, 71·0; H, 10·8. $C_{20}H_{36}O_4$ requires C, 70·6; H, 10·6%). The foregoing ester (1·0 g.) dissolved on warming at 100° with 2N-NaOH (25 ml.) and after 30 min. the solution was cooled, acidified with dil. HCl and extracted with ether. After removal of the solvent the product was distilled (0·7 g., b.p. 115°/0·002 mm.) and solidified. It had m.p. 43–45°, undepressed on admixture with the acid $C_{10}H_{20}O_3$ described by Cartwright (1955).

By prolonged treatment of the acid $C_{10}H_{20}O_3$ with hot conc. HCl the cyclic ester was produced and examined in the way described above. Neutral products from various acylation experiments submitted to similar procedures gave high yields of the acid $C_{10}H_{20}O_3$.

Synthesis of racemic and optically active serratamic acid. DL-Serine (1·0 g.) in methanol (25 ml.) was saturated at room temp. with dry HCl and the solution refluxed for 3 hr. and then evaporated to dryness in a vacuum. It was redissolved in methanol and refluxed for a further period of 1 hr. and the solvent again removed *in vacuo*, the solid dissolved in water (0·5 ml.), covered with ether (25 ml.) and anhydrous K_2CO_3 (10 g.) added in portions with shaking. The ethereal solution of DL-serine methyl ester was decanted and dried over anhydrous K_2CO_3 and used as described below.

DL-3-Hydroxydecanoic acid (1·5 g.) and pyridine (0·63 g.) in dry $CHCl_3$ (25 ml.) was treated under reflux for 15 min. with freshly distilled acetyl chloride (0·63 g.) and the solvent removed at low temp. *in vacuo*. The product was dissolved in ether, washed with water and dried over anhydrous Na_2SO_4, and the solvent removed and replaced by $CHCl_3$ (25 ml.); pure freshly distilled $SOCl_2$ [9·6 ml. of a 10% (w/v) solution in $CHCl_3$] was added and the mixture refluxed for 0·5 hr. and the solvent then removed *in vacuo*. The product was dissolved in dry ether (25 ml.) containing pyridine (0·75g.) and added to the ethereal solution of DL-serine methyl ester prepared above. After standing overnight the ethereal solution was decanted from the pyridine hydrochloride, washed with dil. HCl, $NaHCO_3$ solution and water, dried over Na_2SO_4 and evaporated. The residual oil was shaken for 24 hr. at room temp. with N-NaOH (20 ml.), the resulting solution being acidified carefully with dil. HCl. The addition of ether caused solidification of part of the oily product, which on filtration gave *racemic serratamic acid* or DL-N-(3-*hydroxydecanoyl*)*serine* (0·5 g.), m.p. 142–144° (Found, in purified product, m.p. 144°, from ethyl acetate: C, 57·1; H, 9·3; N, 4·8%; equiv. wt. 278. $C_{13}H_{25}O_5N$ requires C, 56·8; H, 9·1; N, 5·1%; equiv. wt. 275).

By exactly similar procedures serratamic acid was synthesized from (*a*) the L-serine and D-3-hydroxydecanoic acid moieties obtained from hydrolysis of serratamic acid itself, and (*b*) from commercial L-serine and D-3-hydroxydecanoic acid obtained in the resolution of DL-3-hydroxydecanoic acid described above. The product, N-(D-3-*hydroxydecanoyl*) L-*serine*, crystallized from ethyl acetate or water in needles, m.p. 136–137°, undepressed on admixture with natural serratamic acid, m.p. 137–138° (Found: C, 57·0; H, 9·4; N, 4·9%; equiv. wt. 275. $C_{13}H_{25}O_5N$ requires C, 56·8; H, 9·3; N, 5·1%; equiv. wt. 275).

Chloroserratamic acid methyl ester. The non-volatile residue which was insoluble in $NaHCO_3$ solution from the hydrolysis of serratamic acid (2·0 g.) by methanol–HCl (Cartwright, 1955) solidified and crystallized from light petroleum (b.p. 60–80°) in needles (0·2 g.), m.p. 91°. (Found: C, 54·9; H, 8·7; Cl, 11·5; N, 4·7. $C_{14}H_{26}O_4NCl$ requires C, 54·6; H, 8·5; Cl, 11·5; N, 4·6%).

Deoxyserratamic acid methyl ester. The foregoing chloro compound (0·2 g.) was dissolved in methanol (5 ml.) and shaken in an atmosphere of H_2 in the presence of Pd-charcoal catalyst (10%, w/w, 0·05 g.) and 2N-NaOH (0·3 ml.). In 10 min. 14 ml. of gas was absorbed (approx. theoretical) and the catalyst was removed, the solution diluted with water and the product extracted into ether. After shaking with $NaHCO_3$ solution the ether solution was dried and evaporated, leaving a residue (0·1 g.) which crystallized from light petroleum (b.p. 40–60°) in rosettes of needles, m.p. 57–58° (Found: C, 61·3; H, 10·3; N, 4·8. $C_{14}H_{27}O_4N$ requires C, 61·5; H, 10·0; N, 5·1%).

Hydrolysis was effected by heating on the water bath with conc. HCl (2·0 ml.) for 30 min. After dilution with water and shaking with ether the aqueous layer was separated and concentrated *in vacuo*. A portion of the product thus

obtained was applied to a paper along with L-serine and authentic α-alanine and the chromatogram run in 80% phenol. Development with ninhydrin showed the product to have the same R_F value as α-alanine.

2:3-Epoxydecanoic acid. Freshly distilled octyl aldehyde (5·7 g.) and ethyl chloroacetate (5·5 g.) in dry ether (100 ml.) were added to sodium methoxide (from 1·0 g. of Na) suspended in dry ether. After standing for 48 hr. at room temp. water was added, and the ether layer was separated and dried over anhydrous Na_2SO_4. The oil left after removal of the solvent was purified by vacuum distillation, yielding ethyl 2:3-epoxydecanoate (6 g., b.p. 90–93°/0·1 mm.). Saponification rapidly occurred on the addition of an excess of 12% (w/v) KOH in methanol at room temp., with the precipitation of a potassium salt. The mixture was left overnight, diluted with water and carefully acidified with dil. HCl, when the 2:3-*epoxydecanoic acid* separated as an oil which solidified on standing and was crystallized from light petroleum (b.p. 40–60°) in plates, m.p. 59° (Found: C, 64·6; H, 9·5%; equiv. wt. 184. $C_{10}H_{18}O_3$ requires C, 64·5; H, 9·7%; equiv. wt. 186). This substance depressed the m.p. of 3-hydroxydecanoic acid, which could possibly have also arisen in the condensation. A solution of the acid in saturated $NaHCO_3$ solution precipitated a sodium salt on standing, and this behaviour was not observed under similar conditions with 3-hydroxydecanoic acid.

Fission of the epoxide ring and regeneration of 2:3-epoxydecanoic acid. (1) The acid (0·5 g.) was dissolved in conc. HCl (20 ml.) and the mixture heated with frequent shaking on a water bath for 30 min. After dilution with water and extraction with ether, acidic material was removed from the organic phase by saturated $NaHCO_3$ solution. Acidification of the alkaline layer with dil. HCl yielded *chlorohydroxydecanoic acid* (0·3 g.), which crystallized from light petroleum (b.p. 40–60°) in needles, m.p. 93° (Found: C, 53·9; H, 8·7; Cl, 15·6. $C_{10}H_{19}O_3Cl$ requires C, 54·0; H, 8·6; Cl, 15·9%).

(2) The chloro acid (0·2 g.) was added to a solution of KOH in methanol (20%, w/v; 2 ml.). Solid rapidly separated from the mixture, which was stored at room temp. for 2 hr. After dilution with water and acidification the product was separated in ether and crystallized from light petroleum (b.p. 40–60°) in plates, (0·15 g.), m.p. 58–59°, undepressed on admixture with 2:3-epoxydecanoic acid prepared above.

Derivatives of 2-hydroxy-2-methylnonanoic acid. *p*-Nitrobenzoyl chloride (0·1 g.) was added to 2-hydroxy-2-methylnonanoic acid (0·1 g.) in dry pyridine (2 ml.) and the mixture kept at 56° for 18 hr. To the cooled solution dil. HCl was added until the odour of pyridine was no longer apparent, and the solid material was filtered off, washed with $NaHCO_3$ solution and water, dried and crystallized from light petroleum (b.p. 80–100°). 2-*Methyl*-2-p-*nitrobenzoyloxynonanoic acid* (0·15 g.) separated in cream-coloured needles, m.p. 137° (Found: C, 60·5; H, 7·0; N, 4·0. $C_{17}H_{23}O_6N$ requires C, 60·5; H, 6·9; N, 4·2%). The p-*bromophenacyl ester* of 2-*hydroxy-2-methylnonanoic acid* was prepared in 80% yield by refluxing for 1 hr. equimolecular quantities of the sodium salt and *p*-bromophenacyl bromide in ethanol. It formed felted needles, m.p. 111°, from light petroleum. (Found: C, 56·2; H, 6·5; Br, 20·7. $C_{18}H_{25}O_4Br$ requires: C, 56·1; H, 6·5; Br, 20·8%).

SUMMARY

1. The acid, $C_{10}H_{20}O_3$, from hydrolysis of serratamic acid has been equated with D-3-hydroxydecanoic acid.

2. Racemic serratamic acid and serratamic acid have been synthesized and shown to be the optically inactive and active forms respectively of *N*-(3-hydroxydecanoyl)serine.

3. DL-3-Hydroxydecanoic acid has been resolved by fractional crystallization of cinchonidine salts.

4. 2:3-Epoxydecanoic acid has been synthesized.

5. The infrared spectra of serratamic acid and several oxygenated decanoic acids have been recorded.

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Synthesis of Cellulose by *Acetobacter xylinum*

3. SUBSTRATES AND INHIBITORS*

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Earlier communications described a microassay of cellulose (Schramm & Hestrin, 1954*a*) and the preparation of *Acetobacter xylinum* as a fibril-free, stable suspension of washed non-proliferating cells of high cellulose-synthesizing potency (Hestrin & Schramm, 1954). It was hoped that with the aid of these tools an effective approach could be made to unsolved problems in the chemistry and mechanics of cellulose production, and indeed that it might not be difficult to effect a separation of the cellulose-synthesizing enzyme system from the structure of the cell. However, this hope has not been fulfilled.

* Part 2: Hestrin & Schramm (1954). For a preliminary communication on some experiments described in the present paper and results obtained in a tracer analysis of cellulose production, see Schramm, Gromet & Hestrin (1957).

In the absence of a soluble preparation of cellulose-synthesizing enzyme, the investigation has been restricted to the consideration of the behaviour of whole cells in suspension.

The substrate range of the production of cellulose and carbon dioxide in a suspension of washed *A. xylinum* cells, carbon balances and some effects of inhibitors on the pattern of carbohydrate metabolism are reported here. The oxidation of glucose via gluconate to oxogluconates (OGn) and conversions of hexoses (glucose, fructose) and gluconate into hexose phosphate (hexose-P) and carbon dioxide in a cell-free extract of this bacterium are described in the next paper (part 4; Gromet, Schramm & Hestrin, 1957).

MATERIALS AND METHODS

Preparation of cell suspension. The strain of *A. xylinum* was the same as that employed in earlier investigations (Hestrin & Schramm, 1954; Schramm & Hestrin, 1954*a*). Except as otherwise indicated, washed glucose-grown cells were used. Mass cultures were grown at 30° on shallow layers of quiescent liquid contained in aluminum pans under loose covers.

The composition of the glucose medium was as follows: glucose 2%, Marmite 0·25%, yeast extract (Difco) 0·25%, bactopeptone (Difco) 0·5%, dipotassium phosphate 0·1%; pH 7. The crop was collected after 36 hr. This choice of time was of importance, as a rapid decline in the cellulose-synthesizing activity accompanied ageing of the cells in this culture medium.

The fructose medium was of the following composition: fructose 2%, yeast extract (Difco) 0·4%, bactopeptone (Difco) 0·3%, L-asparagine 0·1%; pH 5. After the cells had been maintained on this medium for several passages, they were grown on it on a larger scale. The crop was collected after incubation for 48 hr.

Cells were removed from the pellicles as described previously (Hestrin & Schramm, 1954). The cells were washed first in a dilute solution (pH 5) containing 32 mM-sodium phosphate and 16 mM-citrate, and then in successive changes of water. All the operations were carried out in the cold.

The washed cells were stored at −20° as a lyophilized powder ('freeze-dried cells'). In these conditions their cellulose-synthesizing power declined slowly, generally by less than 30% in the first 2 weeks. Cells suspended in the phosphate–citrate buffer (pH 5) could be stored for several weeks with good retention of activity in the form of a frozen cake ('frozen cells').

Composition of test system. Reaction mixtures contained cells, substrate, oxygen and an appropriate buffer (e.g. sodium citrate solution, pH 6·4) in a closed vessel at 30±0·2°. The mixture was agitated by shaking in a water bath at 100 oscillations/min. Synthesis of cellulose was stopped at any desired time by flooding the mixture with ether.

Substrates. β-Glucose 1-phosphate (β-G 1-P) was synthesized enzymically from maltose (Fitting & Doudoroff, 1952). The enzyme preparation was a maltose phosphorylase from a strain of *Neisseria meningitidis* (type I).

5-Oxogluconate (5-OGn), synthesized by Killiani's method (Barch, 1933), was purified by successive recrystallizations. Calcium 2-oxogluconate (2-OGn) was a pure preparation kindly given to us by Dr B. Horecker. Uridine diphophoglucose (UDPG) was a preparation of medium purity kindly given to us by Dr L. Leloir. Sedoheptulose was in equilibrium with sedoheptulosan (Ujejski & Waygood, 1954). Ribose 5-phosphate (R 5-P) was separated from a partial hydrolysate of adenosine monophosphate (Khym & Cohn, 1953). Glucose 4-phosphate (G 4-P) was kindly given by Dr F. Reithel. It was impure, but as only a small amount was available we used it directly. A specimen of polylysine was kindly provided by Dr E. Katchalski. Other compounds were from commercial sources. Barium and calcium salts were converted before use into their soluble Na^+ ion forms.

Quantitative analytical methods. The gas exchanges were measured manometrically. Carbon dioxide in the gas phase was removed continuously by alkali in a central cup. In the suspension of the washed fresh or freeze-dried cells, the endogenous rates both of respiration and cellulose synthesis were negligible or nil. To obtain values of cellulose synthesis, the amount of cellulose initially present was deducted from the total amount found after incubation of cells with substrate. The initial cellulose values were less than 20% of the amounts of cellulose formed during the incubation with glucose.

Reducing power (as glucose) was determined with Somogyi's copper reagent (Nelson, 1944), cellulose by our micro-method (Schramm & Hestrin, 1954*b*), 2-OGn by its reaction with *o*-phenylenediamine (Lanning & Cohen, 1951) and 5-OGn with 1-methyl-1-phenylhydrazine sulphate (Schramm, 1956). Relative reducing powers of glucose, 2-OGn and 5-OGn towards the copper reagent were 1·00:0·75:0·55. In the presence of OGn glucose was measured iodometrically (Macleod & Robison, 1929). Pyruvate was estimated according to Friedemann & Haugen (1943).

Chromatographic procedure. The chromatograms were prepared on Whatman no. 1 filter paper. Solvent systems were (1) butanol–acetic acid–water (4:1:5, by vol.), (2) methanol–ethanol–water (9:9:2, by vol.) and (3) phenol saturated with water. The development times were 54, 24 and 32 hr. respectively.

Reducing substances were detected on paper with alkaline dinitrosalicylate (Jeanes, Wise & Dimler, 1951). Aniline oxalate (Horrocks, 1949) gave characteristic colour reactions distinguishing respectively glucose, 2- and 5-OGn (see Table 3). Acid substances were located with bromophenol blue indicator. Acetic acid, separated by distillation, was identified on the basis of mobility on paper in a solvent system (Jones, Dowling & Skraba, 1953) containing butanol–water–diethylamine (100:15:1, by vol.).

EXPERIMENTAL AND RESULTS

Survey of substrates

Ranges of substrate for bacterial cellulose production have been described previously by several investigators (Brown, 1886; Tarr & Hibbert, 1931; Khouvine, 1936; Hestrin, Aschner & Mager, 1947; Kaushal & Walker, 1951; Barclay, Bourne, Stacey & Webb, 1954). As almost all the earlier work was

Table 1. *Substrate range of washed glucose-grown cells of* A. xylinum

Activities are calculated on a basis relative to that of glucose (=10) for the same conditions of substrate concentration and pH of medium. Reaction rates [μmoles/mg. of cells (dry wt.)/hr.] with glucose ranged, depending on assay conditions and cell preparation, within the following limits: uptake of O_2 and formation of CO_2, 1·5–5 (measured within the interval 0–30 min.); formation of cellulose monomer, 0·1–0·4 (measured at the end of the interval 0–1 or 0–2 hr.). The reaction mixture contained 5–10 mg. (dry wt.) of cells. The final volume was 2 ml. with air as gas phase and 25 mM-citrate + 50 mM-sodium phosphate as buffer in the experiments with fresh cells. and 20 ml. with O_2 as gas phase and as buffer 20 mM-sodium citrate at pH 6·4 and 40 mM-2-amino-2-hydroxymethylpropane-1:3-diol at pH 7·5 in the experiments with freeze-dried cells. The rates of gas exchange were all maximal or nearly so from the outset of reaction and did not fall off markedly within the period of measurement. Values of the respiratory quotient (R.Q.) were determined for the reaction interval 0–30 min.

			Activity of cells (glucose = 10)							
			Fresh cells				Freeze-dried cells			
Compound	Concn. (mM)	pH	Cellulose	O_2	CO_2	R.Q.	Cellulose	O_2	CO_2	R.Q.
Gluconate	5	6·4	—	4	7	1·0	4	4	8	1·0
2-OGn	5	6·4	—	—	—	—	0	0·5	1	1·0
	20	7·5	—	—	—	—	0	—	—	—
	7·5	6·4	4*	6	11	1·0	—	—	—	—
	7·5	4·0†	1*‡	2	3	1·0	—	—	—	—
5-OGn	7·5	6·4	1*‡	3	5	1·0	—	—	—	—
	7·5	4·0†	7*	7	11	1·2	—	—	—	—
	20	6·4	—	—	—	—	0	—	—	—
	20	7·5	—	—	—	—	0	—	—	—
Fructose	160	6·4	—	—	—	—	0§	0	0	—
Glycerol, DHA	40	6·4	—	—	—	—	1	—	—	—
		7·5	—	—	—	—	10	—	—	—
Cellobiose, maltose	160	6·4	—	—	—	—	1	—	—	—
G 6-P, α-G 1-P	40	6·4	—	—	—	—	0‖	—	—	—
Other P-esters¶	40	6·4	—	—	—	—	0	—	—	—
Other compounds**	160	6·4	—	—	—	—	0	—	—	—

* This number was calculated on the basis of values found at a reaction time of 7 hr.

† At this pH, though not at pH 6·4, citrate probably contributed appreciably to the observed respiration, values of O_2 uptake and CO_2 formation with citrate alone being 1 and 2 respectively, at pH 4 as compared to ⩽ 0·5 at pH 6·4. Values presented in the Table are after deduction of the citrate rates from the rate observed in the presence of added substrate.

‡ Trace of cellulose. § With another preparation of cells, the value found was 2.

‖ Orthophosphate was not found to have been released into the medium.

¶ β-G 1-P, UDPG, G 4-P (crude), fructose 1:6-diphosphate (F 1:6-P), and R 5-P.

** Pentoses (D-xylose, D-ribose, L-arabinose), some hexoses (D-mannose, D-galactose, L-sorbose), sedoheptulose, several saccharides (sucrose, αα-trehalose, lactose, Schardinger α-dextrin, partial hydrolysate of dextran, starch; mM is expressed as to hexose), sorbitol, L-ascorbate, glycerophosphate, ethanol, acetate, pyruvate and lactate.

Table 2. *Substrate range of washed fructose-grown cells*

Reaction mixtures (2 ml.) contained 15 mg. (dry wt.) of cells and 7·5 mM-substrate in 20 mM-sodium citrate buffer, pH 6·4. The gas phase was air. For explanation of units of measurement, see legend to Table 1.

	Reaction rates		
Added substrate	Cellulose*	O_2	CO_2
Glucose	10 (10)	10	10
Acetate†	0	3	—
Pyruvate	0	4	6
Fructose	10 (11)	5	6
Gluconate	— (14)	—	—

* Values in parentheses give results found in a parallel experiment in which 300 mM-substrate was employed with O_2 as gas phase.

† Completely oxidized to CO_2.

done on growing cultures it did not distinguish between a possible effect of a substrate on cell growth from one on cellulose production. By using washed non-proliferating cells in the present investigation, this source of ambiguity could be eliminated.

Cellulose was formed by washed cells of *A. xylinum* from dihydroxyacetone (DHA), glycerol, fructose, glucose, gluconate, 2-OGn and 5-OGn (Tables 1 and 2). The range of substrates yielding CO_2 on oxidation by these cells at pH 6·4 not only comprised cellulose-forming substrates but also others, such as acetate (tested in citrate buffer) and pyruvate, succinate, fumarate, L-malate, oxaloacetate and α-oxoglutarate (all tested in phosphate buffer). Citrate, like *iso*citrate, gave no cellulose at either pH 6·4 or 4·0; it was oxidized at pH 6·4

sluggishly, if at all; however, its oxidation rate was significant at pH 4 (Table 1). Although acetate and lactate have not been converted into cellulose either in a growing culture of *A. xylinum* (Greathouse, Shirk & Minor, 1954) or in the washed-cell suspension, they have been found to be converted into cellulose in a growing culture of *A. acetigenum* (Bourne & Weigel, 1954; Stacey, 1954).

The range of substrates for both cellulose and CO_2 formation depended on the cell history and on the conditions of the assay. An adaptive effect of carbohydrate composition of the growth medium on the substrate range is illustrated by the behaviour of the washed cell towards fructose (cf. Tables 1 and 2). Effects of pH of the assay system are illustrated by the findings with citrate, 5-OGn, DHA and DHA-forming glycerol; a restrictive effect of freeze-drying is shown by 2-OGn (Table 1).

Carbon balances of hexose transformation

Freeze-dried and fresh cells both formed the same products from glucose (Table 3). These were (*a*) native high-polymer cellulose found in the extracellular phase (Hestrin & Schramm, 1954), (*b*) CO_2, (*c*) gluconate (see also Brown, 1886) which could be demonstrated in the medium within minutes after contact of the cells with glucose but could no longer be detected once rapid production of cellulose had come to an end, and (*d*) two oxogluconates identified as 2- and 5-OGn. In the suspension of freeze-dried cells and in one of fresh cells in glucose medium, the ratio 2-OGn:5-OGn was 2:1 (see also Kulka & Walker, 1954).

The amounts of cellulose formed from hexose (glucose, fructose) were not altered noticeably when CO_2 was removed continuously from the gas phase. On the assumption that CO_2 production, like that of cellulose, does not depend on CO_2 in the gas phase, Warburg's direct method could be used as a means of estimating CO_2 production. Carbon balances drawn up in this manner are collected in Table 4. The analytical recoveries of carbon in the experiments with glucose are $\geqslant 84\%$, i.e. within the probable range of the accumulative error of the analyses. Within these limits, the balance sheets of the glucose transformation could be described by the following empirical equation:

$$(s)\ \text{glucose} + (6p+q)\ O_2 \rightarrow (6p)\ CO_2 + (q)\ \text{OGn} + (r)\ \text{cellulose monomer} + (6p+q+r-1)\ H_2O, \quad (1)$$

where $s=p+q+r$ (moles) and r is the degree of polymerization of the cellulose.

A carbon balance for fructose is included in Table 4. In this experiment, the substrate was in the form of [1-^{14}C]fructose. The reported analytical deficit (11%) is only slightly greater than the probable cumulative error of the analytical methods used. At the time of the drawing up of the balance sheet, the copper-reducing power of the medium was 5% of the initial value and the quotient '^{14}C/reducing power' was 200% of that of fructose. [The persisting reducing product in this reaction mixture could conceivably have been a disaccharide (cf. Wright & Walker, 1955)]. In accordance with the conclusion that the only oxidation product formed and accumulated in substantial amount in this system was CO_2, the R.Q. of fructose was found to be 1·0 throughout the reaction.

In different batches of fresh and dried cells there were marked variations in the percentage of glucose converted into cellulose, of glucose converted into OGn, ratio of OGn to CO_2, ratio of cellulose to OGn, ratio of cellulose to glucose converted into CO_2 (r/p) and ratio of cellulose to O_2 uptake [$r/2(q+6p)$] (Table 4). Since freeze-dried cells oxidized OGn much more slowly than they did glucose or gluconate, the net amounts of OGn found in the suspensions of freeze-dried cells in glucose solution are probably only slightly less than the gross amounts.

Table 3. *Hexonates formed by* A. xylinum *from glucose and gluconate*

The test mixture contained 15 mg. of freeze-dried cells and 50 mg. of substrate in 5 ml. of water under O_2; the pH was maintained at 5 by intermittent alkali addition. The reaction time was 2 hr. For analysis the reaction mixtures were cleared by centrifuging and passed through a cation-exchange column (Amberlite IR 100, H^+). The effluents were reduced in volume by freeze-drying and then examined by paper chromatography. A control experiment in which the *A. xylinum* cell suspension was incubated without substrate gave negative reactions in all the tests listed in the Table.

	Properties of compound						Formation of compound from substrates	
	R in solvents†							
Compound	1	2	3	Acid group	Free reducing group	Colour with aniline oxalate	Glucose	Gluconate
Glucose	1·00	1·00	1·00	−	+	Brown		
2-OGn	0·55	0·75	0·55	+	+	Red	+	+
5-OGn	1·32	0·60	0·61	+	+	Yellow	+	+
Gluconate	1·05	0·56	0·82	+	−	None	+	
2:5-Dioxogluconate*	0·55	.	.	+	+	Green–yellow	−	−

* Data cited from Katznelson, Tanenbaum & Tatum (1953).

† See under Materials and Methods.

Table 4. *Carbon balances of aerobic transformation of glucose and fructose*

Reaction mixtures (2 ml.) varied as to composition within the following ranges: cells, 8–15 mg. (dry wt.); substrate, 2·5–7·5 mM; buffer, 4–20 mM-sodium citrate, pH 6·4. The gas phase was O_2. The analysis was carried out at the end of 4–5 hr. of reaction. At this time, originally added substrate and intermediately formed gluconate had been consumed, the cellulose synthesis was at a standstill and the rate of CO_2 formation had recently fallen markedly. For the significance of *p*, *q*, and *r* see eqn. (1).

Expt. no.	Cells: Batch	Cells: Condition	Sugar in growth medium	Substrate	Analytically determined products (initially added hexose = 100): CO_2 (*p*)	2-OGn + 5-OGn (*q*)	Cellulose (*r*)
1	*a*	Freeze-dried	Glucose	Glucose	12	32 + 20	20
2	*b*	Freeze-dried	Glucose	Glucose	21	21 + 9	33
3	*c*	Fresh	Glucose	Glucose	40	12 + 5	46
4*	*d*	Fresh	Glucose	Glucose	58	5†	40
5	*e*	Fresh	Fructose	Glucose	70	3†	27
6	*e*	Fresh	Fructose	Fructose	61	.	28

Calculations

Expt. no.	Total analytical recovery of added substrate (carbon, %)	Cellulose monomer / CO_2 (as glucose) (r/p)	Cellulose monomer / Oxygen atom $[r/2(q+6p)]$
1	84	1·7	0·08
2	84	1·6	0·10
3	103	1·1	0·09
4	103	0·7	0·05
5	100	0·4	0·03
6	89	0·5	0·04‡

* Gas phase: air.

† Calculated from the persisting copper-reducing power of the reaction mixture on the basis of the assumption that the ratio '2-OGn:5-OGn' was 2.

‡ Calculated on the assumption that $q=0$.

Thus the listed values of the ratio q/p of freeze-dried cells can be taken as a rough index of relative activity of alternate metabolic pathways. Increases in q/p are known to accompany the ageing of *Acetobacter* spp. in culture (Butlin, 1938; Kluyver & Boezaardt, 1938). In a washed *A. xylinum* cell, this phenomenon appears to have been produced by freeze-drying, probably because such treatment selectively injured a component of the mechanism of OGn oxidation. As no pair of the *p*, *q* or *r* terms observed a constant relationship in this series of experiments, it seems probable that *q* and *p* comprised components which are facultatively dissociable from *r*.

Gluconate as an intermediate of glucose metabolism

Gluconate gave the same products of reaction as glucose (Table 3). It was itself formed much more rapidly from glucose than was either CO_2 or cellulose. Thus at a low glucose concentration ($\leqslant 10$ mM), the R.Q. of glucose oxidation by freeze-dried cells was initially low ($<0·5$) for a short time (Fig. 1), but at high glucose concentration (100 mM) in this system the period of low R.Q. persisted for at least 1 hr. At the higher concentration of substrate the amount of gluconate (total non-volatile acid minus total OGn) formed in 1 hr. exceeded several-fold the equivalent of the sum of all the other reaction products (CO_2, cellulose and OGn). With a suspension of fresh cells (Expt. 4 in Table 4) and 2·5 mM-glucose, the net amounts of gluconate, CO_2 and cellulose formed were found to be respectively (as % of initial glucose): 60, 7 and 8 after 10 min.; 0, 58 and 40 after 5 hr. Such data require that the bulk of cellulose and

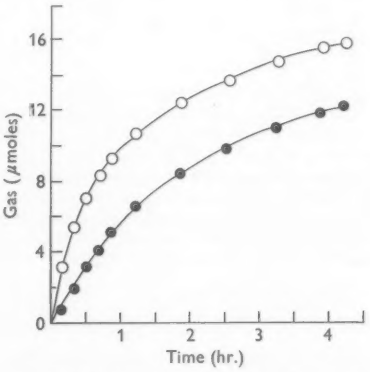

Fig. 1. Course of oxidation of glucose by freeze-dried cells. Reaction mixture (2 ml.) contained 15 mg. of cells, 5 mM-glucose and 10 mM-sodium citrate, pH 6·4. The gas phase was O_2. ○, O_2 uptake; ●, CO_2 evolution.

CO_2 were formed via gluconate formed intermediately in these cell preparations.

Time-courses of the production of cellulose from added glucose and gluconate are compared in Fig. 2. It can be seen that gluconate was inferior as a substrate to glucose during the first hour of reaction but that the subsequent course and magnitude of the synthesis from both 'glucose' and gluconate became practically identical. Since glucose supported a more rapid initial synthesis than did gluconate, it could be supposed either that gluconate entered the cells less rapidly than did glucose, or that gluconate was a facultative but not an obligatory intermediate of synthesis from glucose.

Anaerobic systems

Cellulose was not synthesized under N_2 as gas phase in systems containing glucose and added electron acceptors other than O_2 (Table 5). In the absence of an added electron acceptor, glucose was not attacked. The addition of several dyes (e.g. 2:3:5-triphenyltetrazolium salt and 2:6-dichlorophenolindophenol), which are known to enter into reaction with flavins, allowed the rapid oxidation of glucose and gluconate but did not permit formation of either cellulose or CO_2. The ability to oxidize gluconate by the action of dyes distinguishes the OGn-forming system in *A. xylinum* from that in some other OGn-forming bacteria (cf. Wood, 1955).

The addition of adenosine triphosphate (ATP) failed to initiate the anaerobic synthesis of cellulose from glucose or gluconate, nor was the ATP hydrolysed [cells in 2-amino-2-hydroxymethylpropane-1:3-diol (tris) buffer at pH 7 with added Mg^{2+} ions]. An introduction of O_2 into this system promptly initiated cellulose synthesis.

Pyruvate was rapidly metabolized anaerobically, affording 1 mole of CO_2, 0·5 mole of acetate (identified chromatographically) and, presumably, 0·5 mole of ethanol (cf. Stephenson, 1949)/mole of utilized pyruvate.

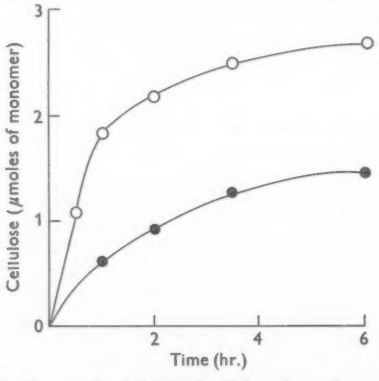

Fig. 2. Course of synthesis of cellulose from glucose and gluconate by freeze-dried cells. Reaction mixtures (2 ml.) contained 10 mg. of cells, 5 mM-substrate (glucose or gluconate) and 10 mM-sodium citrate, pH 6·4. Gas phase was O_2. ○, Glucose; ●, gluconate.

Modification of the pattern of glucose metabolism by inhibitors

Compounds that have been tested for ability to inhibit cellulose synthesis from glucose are listed in Table 6 in their probable order of descending potency.

Fluoride. Cellulose synthesis from glucose in citrate medium was blocked by 10 mM-fluoride both in the presence and absence of added orthophosphate (5 mM) (Table 7). Carbon dioxide formation in this system was retarded significantly, but not blocked, by fluoride acting in the absence of added orthophosphate. However, no effect of fluoride on CO_2 production occurred in the presence of added orthophosphate (Fig. 3; cf. also, Hestrin *et al.* 1947). The oxidation of glucose by cells poisoned by fluoride in citrate medium with added orthophosphate proceeded smoothly until practically all of the glucose had been converted into CO_2.

Table 5. *Anaerobic oxidation of glucose and gluconate by addition of electron acceptors to a suspension of cells*

Reaction mixtures (3 ml.) contained 8 mg. (dry wt.) of fresh cells, mM-substrate (glucose or gluconate) and 10 mM added electron acceptor in 10 mM-sodium citrate, pH 6·4, under N_2. Without added substrate a relatively slow reduction of added electron acceptors occurred. ++, Rapid reaction; ± very sluggish reaction (slightly faster than that of system without added substrate); 0, no reaction. In a similar experiment with freeze-dried cells, all the alternate acceptors listed below were rapidly reduced both by glucose and gluconate without attendant cellulose synthesis. When O_2 was introduced into these systems, rapid synthesis of cellulose occurred.

Substrate	Electron acceptor	Activity: Reduction of acceptor	Activity: Formation of cellulose	Activity: Formation of CO_2
Glucose	2:6-Dichlorophenolindophenol	++	0	0
Gluconate	2:6-Dichlorophenolindophenol	++	0	0
Glucose	2:3:5-Triphenyltetrazolium	++	0	0
Gluconate	2:3:5-Triphenyltetrazolium	++	0	0
Glucose	Ferricyanide	++	0	±

Orthophosphate by itself did not alter the rates and yields of the oxidations and syntheses. Fluoride in the system without added orthophosphate brought about a significant elevation in the OGn value, attended by an almost fourfold rise in the value of the 'OGn:CO_2' ratio (Table 7).

Table 6. *Inhibitors of cellulose synthesis*

Reaction mixtures (20 ml.) contained 10 mg. of freeze-dried cells, 100 mM-glucose, 20 mM-sodium citrate, pH 6·4, and inhibitors as listed. The gas phase was O_2. The reaction time was 60 min.

	Concn. (mM) of inhibitor causing inhibition of cellulose formation		
Inhibition (%)	<20	50±10	>90
Inhibitors			
Cyanide	0·01	0·06	1
p-Chloromercuribenzoate	—	—	1*
N-Ethyl maleimide	—	—	2*
2:4-Dinitrophenol	0·1	0·8	5
Thymol	—	—	5
Fluoride	0·5	3	10
Azide	0·5	4	10
Toluene†	—	5	—
Arsenate	1	10	—
Iodoacetate	2*	10	—
Formate	—	10	—
Acetate	5	30	50‡
Pyruvate	—	70	50‡
Hydroxylamine§	5	100	—
Other compounds‖	10	—	—

* With fresh cells and with 50 mM-sodium phosphate, pH 6·8, as buffer under air as gas phase.

† Cellulose synthesis was also inhibited by other narcotics (ether, chloroform).

‡ With fresh fructose-grown cells and with 5 mM-fructose as substrate, at pH 6 under air as gas phase.

§ pH 6·8.

‖ Malonate, arsenite (50 mM), 8-hydroxyquinoline, ethylenediamine tetra-acetate, inorganic ortho- and pyrophosphate, tetraethyl pyrophosphate; Na^+, K^+, Mg^{2+} and Mn^{2+} ions (with Cl^- ions at 40 mM). In addition it has been found that polylysine (0·01%, w/v), some neutral detergents (sorbitan trioleate and polyoxymethylsorbitan monooleate, 2%, w/v) and streptomycin (5000 units/ml.) failed to inhibit synthesis significantly.

Arsenate. This anion inhibited cellulose synthesis. In accord with the assumption that the action resulted from competition between arsenate and orthophosphate, exogenous addition of phosphate was found to alleviate or abolish the arsenate effect (Fig. 4). That this effect was specific is shown by the observation that an antagonistic effect on arsenate was not given by glycerophosphate.

2:4-*Dinitrophenol.* Cellulose synthesis was suppressed and CO_2 formation from glucose was

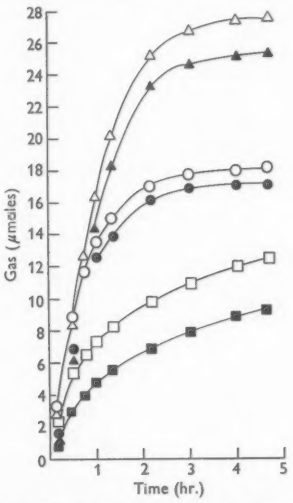

Fig. 3. Effects of inhibitors (fluoride and 2:4-dinitrophenol) on oxidation of glucose by *A. xylinum*. Reaction mixtures (2 ml.) contained 8 mg. of fresh cells, 2·5 mM-glucose and 20 mM-sodium citrate, pH 6·4, with or without inhibitor. Substrate was introduced 60 min. after contact of the cells with inhibitor. ○, ●, O_2 and CO_2 without additions or with 5 mM-orthophosphate (P_i) only; △, ▲, O_2 and CO_2 with 10 mM-fluoride plus 5 mM-P_i; □, ■, O_2 and CO_2 with 4 mM-DNP.

Table 7. *Carbon balance of glucose metabolism by cells acting in the presence and absence of added orthophosphate, fluoride and 2:4-dinitrophenol*

Reaction mixtures are described in Fig. 3. Carbon balances were made at the time of the conclusion of the manometric measurements (reaction time 4·5 hr.).

System			O_2 uptake	Products of reaction (as μmoles of glucose)			Analytical recovery of carbon
Fluoride	P_i§	DPN	(μmoles)	CO_2*	OGn	Cellulose	(%)
−	−	−	18·2	2·9	Approx. 0·3	2·0	104
−	+	−	18·0	2·8	Approx. 0·2	2·1	102
+	−	−	24·2	3·6	1·2†	0·0	96
+	+	−	27·6	4·25	0·5	0·0	95
−	−	+	12·5	1·55	3·2‡	0·0	95

* Actual moles of CO_2 were six times these figures.

† The ratio '2-OGn:5-OGn' was found to be 5.

‡ Calculated from the persisting copper-reducing power on the basis of the arbitrary assumption that the ratio '2-OGn:5-OGn' in the suspension of DNP-poisoned cells, as in normal freeze-dried cells, was 2.

§ P_i=5 mM-orthophosphate.

markedly retarded by 4 mM-2:4-dinitrophenol (DNP) (Table 7). The kinetics of oxidation observed in the presence of the inhibitor were bimodal to both rate and R.Q. (Fig. 3). It can be noted that DNP resulted in marked elevation of both the absolute net amount of OGn formed and of the ratio 'OGn:CO_2' (Table 7). Almost all of the amount of substrate which had not been converted by the end of the experiment into CO_2 was OGn.

Acetate and pyruvate. It has been observed that acetate, not itself incorporated into cellulose, promotes formation of cellulose from glucose in a growing culture (Greathouse *et al.* 1954). Accordingly, it seemed of interest to learn whether added acetate or acetate-forming pyruvate exert a similar effect in a suspension of washed cells. The unexpected finding was then made that synthesis from glucose and fructose in this system is not promoted but rather markedly inhibited by acetate and pyruvate.

As acetate can be oxidized rapidly, a high 'acetate:hexose' ratio seemed desirable to obtain a sustained inhibitory effect. In a typical instance, 50 mM-acetate was incubated with 15 mg. (dry wt.) of fructose-grown fresh cells in 5 mM-fructose. The added acetate suppressed the synthesis during an observation period (2 hr.) which permitted abundant cellulose formation in a control reaction mixture. Further experiments with the view to clarifying this remarkable inhibitory action are intended.

Arsenite. Cells in citrate buffer oxidized glucose and formed cellulose without attendant accumulation of a detectable amount of pyruvate in the medium of a standard-test system. In the presence of 5 mM-arsenite, glucose was still converted into cellulose and presumably therefore was also oxidized, but pyruvate accumulation was not demonstrable.

Fig. 4. Effect of phosphate on the inhibition of cellulose production by arsenate. Reaction mixtures (5 ml.) contained 13 mg. of freeze-dried cells, 150 mM-glucose, 20 mM-sodium citrate, pH 5, and arsenate with and without orthophosphate. The gas phase was O_2. The reaction time was 60 min. ●, ○, 10 and 2 mM-arsenate respectively.

Fluoroacetate. This agent in 10 mM-concentration with an aerobic suspension of cells acting on glucose in 20 mM-sodium citrate solution, pH 5, permitted cellulose formation and did not diminish or may have slightly enhanced the rate of production of CO_2. In the absence of inhibitor, pyruvate and acetate were rapidly and completely oxidized to CO_2. Fluoroacetate (10 mM) prevented the oxidation of acetate and stopped the pyruvate oxidation at a level corresponding to conversion of pyruvate into acetate.

DISCUSSION

In most species of cells, the aerobic metabolism of exogenous substrates sustains attendant reactions of assimilation leading to several products of reaction that are stored intracellularly. In the washed suspension of cells of *A. xylinum*, however, cellulose deposited extracellularly has been shown above to be formed in abundance (up to 45% of all added glucose) and to be the only quantitatively important product of the aerobic assimilation of exogenous glucose (Table 4). On these counts, *A. xylinum* might be regarded as a particularly suitable system for the study of mechanisms by which oxidative energy is harnessed to synthetic needs.

The information available does not suffice to permit the drawing of a detailed energy balance of oxidative assimilation of glucose by *A. xylinum*, but it may help to resolve the problem into more readily manageable components.

The highest observed value of the ratio 'cellulose: oxygen atom' in *A. xylinum* was never greater than about 0·1. A ratio as high as 3 might have been anticipated on the basis of the assumption that the energy of oxidation is channelled in this organism with ideal efficiency into synthesis of high-energy phosphate compounds ($\sim$P) and that the ratio (monomer synthesized from glucose):($\sim$P generated) can be 1. Obviously the process of oxidative assimilation in *A. xylinum* falls far short of this ideal situation. Although the metabolism of ketoses (fructose, DHA) presumably does not involve OGn production, they were able to sustain vigorous cellulose production. Therefore, even if it is admitted that 'direct' oxidative steps in glucose metabolism are able to afford useful energy, the latter need not be regarded as obligatory for the synthesis of cellulose. An analogous assessment can be made of the probable value of pyruvate oxidation as a potential source of useful energy. Since 10 mM-fluoroacetate exerted only a weak inhibitory action on the synthesis of cellulose, whereas it completely

blocked acetate oxidation, it is suggested that the energy provided by pyruvate oxidation was not essential for the synthesis of cellulose.

The attempt to utilize substrate specificity data obtained with whole cells in order to construct a scheme of intermediate metabolism is complicated by the likelihood that selective-permeability factors are at play. That such factors were operative in fresh and even freeze-dried cells is indicated by the finding that some substrates (α-G 1-P, G 6-P, ATP, citrate and *iso*citrate) cleaved by disrupted-cell fractions (Gromet *et al.* 1957; unpublished experiments) were not attacked by suspensions of whole cells tested in medium at pH 6·4. Thus the failure of an added P ester to yield cellulose need not detract from the likelihood that this ester is an intermediate of carbohydrate oxidation and an intracellular precursor of cellulose. Similarly, the incapacity of added ATP to sustain anaerobic synthesis does not indicate that ATP is not involved intracellularly in the channelling of energy. Finally, a non-oxidation of added citrate and *iso*citrate by cells does not deny the possibility that they possess a citrate cycle.

It has been shown above that glucose-grown cells of *A. xylinum* form gluconates (gluconate, 2- and 5-OGn) from glucose and also carbon dioxide and cellulose from these substrates. In addition, fructose-grown cells also formed carbon dioxide and cellulose from fructose. Pyruvate, acetate and citrate-cycle intermediates, though rapidly oxidized to carbon dioxide by these cells (present results and unpublished experiments), gave no cellulose. Freeze-drying completely blocked the formation of cellulose and markedly lowered but did not abolish the formation of carbon dioxide from the oxogluconates, though it left almost intact the ability of the cells to form gluconates from glucose and to convert gluconate and glucose into carbon dioxide and cellulose. To rationalize these results it can be assumed that alternate pathways are available to the washed *A. xylinum* cell for the conversion of exogenous substrates into carbon dioxide and cellulose. A scheme of alternate pathways that takes into account the findings with whole cells, evidence derived from use of specifically labelled glucoses as substrates and observations on the enzymic constitution of a cell-free extract of the cells is presented in the next paper (Gromet *et al.* 1957).

The inhibitors of cellulose synthesis (Table 6) belong to a variety of chemical and physiological categories. They include cytochrome complexants (cyanide, azide), 'uncouplers' (DNP, azide), an analogue (arsenate) of phosphate, a halide (fluoride) which inhibits enolase and phosphatases as well as some other enzyme systems (cf. Aisenberg & Potter, 1955), –SH reagents (*p*-chloromercuribenzoate, *N*-ethylmaleimide), 'narcotics' (toluene, ether, chloroform, thymol) and even some familiar oxidizable substances (pyruvate, acetate). It is apparent that an inhibitor could block synthesis by an effect exerted directly on a step in polymerization, by an effect exerted on a necessary transport mechanism, or by an effect on some step in substrate dissimilation or energy transmission on which synthesis may depend. The information available does not indicate the loci of action of individual inhibitors within the network of interlinked metabolic reactions. The case of fluoride may merit an added comment. As this agent permitted a normal rate of production of carbon dioxide, it probably did not interfere with the production of hexose-P. On the basis of the view that cellulose arises from hexose-P (Schramm *et al.* 1957), the inference can then be drawn that fluoride probably acted within the sequence: intracellular hexose-P → extracellular cellulose.

Probable nature of an intracellular precursor of cellulose

Evidence discussed elsewhere (Schramm *et al.* 1957) has suggested that cellulose arises in *A. xylinum* from the internal hexose-P pool of the cell by a reaction which does not necessarily involve cleavage of a carbon–carbon bond in the hexose radical. The finding (Gromet *et al.* 1957) that *A. xylinum* extract is richly endowed with phosphoglucomutase encourages speculation concerning the possibility that α-G 1-P is an intermediary in the formation of cellulose as of starch. A hypothesis, made particularly attractive by recent findings in green plants (cf. Altermatt & Neish, 1956), suggests that UDPG might be the intermediary of cellulose formation in *A. xylinum*, but this is a view which can neither be clearly supported nor rejected on the basis of the limited information in our possession.

The degree of polymerization of *A. xylinum* cellulose has been shown to be nearly 3×10^3 (Ranby 1952). It is difficult to visualize diffusion of such a large particle across an intact cell wall which exhibits selective impermeability towards much smaller molecules. Moreover, cellulose did not accumulate within the cell interior (Hestrin & Schramm, 1954). Thus if we suppose that cellulose is fully formed within the cell and then expelled, we assume that organelles for the active extrusion of cellulose into the medium exist and that cellulose is probably formed only within them. Such far-reaching assumptions would be unsupported. There remain for consideration two alternatives: (*a*) the exogenous substrate is transformed into extracellular cellulose by an exclusively extracellular sequence of reactions (the term 'extracellular' is used in a sense that comprises either reactions in the medium or on the *outer* surface of the cells); (*b*) the exogenous substrate enters the cell and is converted

into a precursor which is excreted and converted into cellulose extracellularly. Were (*a*) valid, it would be expected that *added* glucose 6-phosphate (G 6-P) and α-G 1-P would be converted into cellulose and carbon dioxide. This was not the case. Hence it is deduced that the transformation of exogenous substrate into cellulose proceeded via an intracellular step (cf. also Hestrin *et al.* 1947). The familiar P-esters are not known to be readily moved into or out of an intact cell. It has been shown above that glucose 1-phosphates (α-G 1-P, β-G 1-P and UDPG) and G 4-P provided exogenously are not converted into cellulose on the outer surface of *A. xylinum*. Exogenously added G 6-P and the pentose-cycle intermediates formed from G 6-P by a fortified crude extract of *A. xylinum* were not converted into cellulose on the outer surface of the *A. xylinum* cell (Gromet *et al.* 1957). The possibility is therefore considered that the immediate cellulose precursor is not itself a P-ester difficult to transport but a derivative in which the P group has been replaced or substituted by a radical whose presence facilitates transport across the cell wall. [This argument may also be pertinent to some other cases (e.g. Mager & Aschner, 1947) in which internal hexose-P is the likely precursor of an extracellularly deposited high polymer.]

Cellulose appears to be deposited in green plants intracellularly. Therefore, in these, the above objection to the view that hexose-P is the *immediate* precursor of cellulose might not necessarily apply. However, very little is known as yet about the chemical mode of synthesis of cellulose (Greathouse, 1953; Brown & Neish, 1954; Edelman, Ginzburg & Hassid, 1955; Shafizadeh & Wolfrom, 1955; Altermatt & Neish, 1956) and mechanics of deposition (Muhlethaler, 1949; Preston & Kuyper, 1951; Ranby, 1952; Steward & Muhlethaler, 1953, 1954; Preston & Astbury, 1954). What little evidence is available does not contradict the possibility that the chemical basis of the synthesis both in green plants and bacteria might be identical.

SUMMARY

1. Hexoses (glucose, fructose), three-carbon compounds (glycerol, dihydroxyacetone) and hexonates (gluconate, 2- and 5-oxogluconate) have been converted into cellulose by the action of washed cells of *Acetobacter xylinum*. The substrate range of the cells was a function of their history and the conditions in which they were assayed. Acetate, pyruvate and citrate-cycle intermediates were oxidized without attendant conversion into cellulose. Phosphate esters (including glucose 6-phosphate, α-glucose 1-phosphate, β-glucose 1-phosphate and uridine diphosphoglucose) presumably failed to penetrate into the cells (fresh and freeze-dried) Such esters, added exogenously, did not form cellulose.

2. The sole product of assimilation formed in substantial amount from glucose was cellulose. Glucose was converted via gluconate into 2- and 5-oxogluconate. It was assimilated to cellulose and oxidized to carbon dioxide facultatively via these gluconates. The highest encountered value of the molar ratio 'cellulose monomer: glucose oxidized to carbon dioxide' was 1·7, i.e. significantly greater than unity. In all instances only a small portion of the total energy liberated by concurrent oxidation of hexose was utilized for cellulose production ('cellulose monomer: oxygen atom' $\leqslant 0{\cdot}1$). Exogenously provided adenosine triphosphate failed to sustain an anaerobic synthesis of cellulose. Electron acceptors other than oxygen permitted restricted oxidation of glucose and gluconate, presumably to oxogluconates, but such oxidation did not enable formation either of cellulose or of carbon dioxide.

3. Synthesis of cellulose from glucose has been blocked by appropriate respiration inhibitors, sulphydryl reagents, 'narcotics' and even by the oxidizable metabolites acetate and acetate-forming pyruvate. Synthesis was retarded by arsenate; this effect was alleviated by added orthophosphate. Whereas 10 mM-fluoroacetate blocked acetate oxidation and caused that of pyruvate to cease at the level of acetate, it permitted both cellulose and carbon dioxide to be formed from glucose. Fluoride (10 mM) blocked the synthesis of cellulose but not the formation of carbon dioxide. 2:4-Dinitrophenol (4 mM) permitted oxogluconate production but blocked the formation of cellulose and retarded that of carbon dioxide from glucose.

4. The findings are in agreement with the suggestion that alternate metabolic pathways are available to the washed *A. xylinum* cell for the conversion of exogenous substrates into carbon dioxide and cellulose and with the hypothesis that an intracellular hexose phosphate is an intermediate in cellulose production.

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Synthesis of Cellulose by *Acetobacter xylinum*

4. ENZYME SYSTEMS PRESENT IN A CRUDE EXTRACT OF GLUCOSE-GROWN CELLS

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The synthesis of cellulose from glucose by washed cells of *Acetobacter xylinum* has been shown to be conditional upon a concurrent oxidative process (Hestrin, Aschner & Mager, 1947; Schramm, Gromet & Hestrin, 1957*a*, *b*). To understand the synthesis, a knowledge of the nature of the oxidative process seemed desirable. Some of the reactions of glucose oxidation that occur in an extract of *A. xylinum* cells are demonstrated in this paper.

METHODS AND MATERIALS

A. xylinum *extracts*. All operations were carried out in the cold room. Cell-free extracts were prepared from washed *A. xylinum* cells harvested from glucose medium (Schramm *et al.* 1957*b*) as follows.

(*a*) '*Alumina*' *extract*: Freeze-dried cells (100 mg.) were triturated in a hand mortar with 300 mg. of alumina (Alumina A-301, Alcoa Ltd., Pittsburgh, Pa., U.S.A.) for 10 min. Then 3 ml. of 0·04 M-sodium citrate buffer, pH 6·4, was added to the mixture. After 10 min. the suspension was centrifuged (3000 *g* for 15 min.) and the packed sediment was re-extracted in 2 ml. of buffer. The supernatant fluids (about 4 ml.) were pooled and partially cleared by centrifuging (3000 *g* for 15 min.). The turbid, slightly pink supernatant fluid was stored at −20°.

(*b*) '*Mickle*' *extract*: Cells (100 mg. dry wt.) were washed in water (10 ml.) and then suspended in 3 ml. of 0·1 M-2-amino-hydroxymethylpropane-1:3-diol (tris) buffer (Cl^-; pH 7·5). Treatment in a Mickle tissue disintegrator (30 min. with about 1 g. of Ballotini glass beads) served to disrupt the cells. This 'homogenate' was centrifuged (3000 *g* for 15 min.), the sediment re-extracted in 2 ml. of buffer and the supernatant fluids (about 4 ml.) pooled and partially cleared (3000 *g* for 15 min.). The product was an opalescent fluid. It formed CO_2 oxidatively from glucose 6-phosphate (G 6-P) even after storage for 1 month at −20°. Such extracts have been obtained from fresh cells, freeze-dried cells and from frozen cells.

Test systems. The temperature was 30±0·2°. Anaerobic experiments were conducted in Thunberg vessels under N_2 at reduced pressure. Conventional Warburg techniques were used in all the manometric determinations, CO_2 being absorbed by alkali in the central cup. Systems with alumina extract were buffered by 0·02 M-sodium citrate, pH 6·4, and the systems with Mickle extract by 0·05 M-tris (Cl^-), pH 7·5. In a Mickle extract acting aerobically on G 6-P, the formation of CO_2 was found to be accelerated by phenazine methochloride (Fig. 5). This compound (final concn. 50–100 μg./ml.) was included in the composition of all the aerobic reaction systems except as otherwise stated specifically in the text.

Reagents. The origin of reagents were as follows: phenazine methochloride (Bios, New York, N.Y., U.S.A.); diphosphopyridine nucleotide (DPN) as a sodium salt (Pabst Laboratories, Milwaukee, Wis., U.S.A.); triphosphopyridine nucleotide (TPN) of 80% purity and a crystalline preparation of adenosine monophosphate (AMP) of muscle (Sigma Chemical Co., St Louis, Mo., U.S.A.); adenosine triphosphate (ATP) and diphosphate (ADP) as sodium salts (Pabst Laboratories); 3-phosphoglycerate (PGA), G 6-P, fructose 6-phosphate (F 6-P) and fructose 1:6-diphosphate (F 1:6-P) as barium salts (Nutritional Biochemical Corp., Cleveland, Ohio, U.S.A.). 6-Phosphogluconate (6-PG) was a barium salt kindly given by Dr B. L. Horecker. Glucose 4-phosphate (G 4-P) was used in the form of a crude preparation kindly given by Dr R. J. Reithel. β-Glucose 1-phosphate (β-G 1-P) and ribose 5-phosphate (R 5-P) were prepared in the laboratory (Schramm *et al.* 1957*b*). Uridine diphosphate glucose (UDPG) was obtained from Dr H. Kalckar. To generate triose phosphate (triose-P) from F 1:6-P a thrice-recrystallized preparation of rabbit-muscle aldolase, kindly given by Dr J. Mager, was used. The barium salts were all converted before use into their soluble sodium forms.

Analytical methods. Samples were clarified by the following methods: acidification with HCl to pH 1 in the estimations of total reducing power by copper reduction and in the enzymic assay of G 6-P; treatment with barium–zinc mixture (Somogyi, 1945) in the assays of non-phosphorylated reducing sugars; 12% (w/v) $HClO_4$ in the estimations of oxogluconates (OGn) by colorimetric procedures; treatment with 5% (w/v) trichloroacetic acid in other cases.

Hexose (as glucose) and heptose (as sedoheptulose) were estimated by reaction with cysteine (Dische, 1955), total ketose (as fructose) with resorcinol (Roe & Papadopoulos, 1954) and pentose (as xylose) with orcinol (LePage, 1949*b*). Heating time with the orcinol reagent was 40 min. F 6-P was estimated by reaction with the resorcinol reagent as described by Roe, Epstein & Goldstein (1949). The colour formed by F 6-P with this reagent was only half as intense as that formed by fructose. Phosphorylated carbohydrate was calculated in all the colorimetric assays as the fraction removed from the test portions by the treatment with barium–zinc mixture. G 6-P was assayed enzymically by reduction of TPN as catalysed by yeast G 6-P dehydrogenase. The latter was prepared from a brewery yeast (Nesher Brewery, Rishon LeZion, Israel) according to Kornberg (1950) and stored as a lyophilized powder. To enhance its specificity, this reagent was always aged in solution (48 hr. at 4°) before use. After ageing, it contained some hexokinase in addition to G 6-P dehydrogenase, but was free from 6-PG dehydrogenase, phosphohexoisomerase and glucose dehydrogenase. Other methods of estimation of carbohydrate have been noted in the preceding communication (Schramm *et al.* 1957*b*).

Orthophosphate (P_i) was determined by the method of Fiske & Subbarow (1925), acid-labile P as ΔP_i in samples held at 97° in N-HCl for 10 min., and alkali-labile P as ΔP_i in samples held at room temperature in N-KOH for 20 min. Adenylic acid was precipitated as its barium salt with ethanol and assayed spectrophotometrically (260 mμ at pH 7). Reduction of DPN and TPN was followed spectrophotometrically at 340 mμ. Protein nitrogen was estimated with a colorimetric reagent (Lowry, Rosebrough, Farr & Randall, 1951). A dried-plasma preparation of known nitrogen content served as the protein standard.

Endogenous rates represented ⩽10% of total readings. Values are reported after having been corrected for endogenous rates, except as otherwise is indicated.

RESULTS

Formation of gluconate from glucose. A cell-free extract of *A. xylinum* oxidized glucose in the presence of phenazine methochloride without attendant evolution of CO_2. The O_2 uptake was 0·5 mol.prop. (Fig. 1). Gluconate was not oxidized.

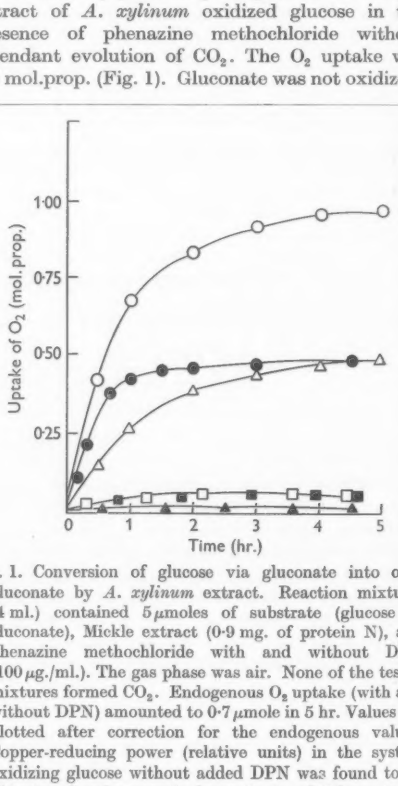

Fig. 1. Conversion of glucose via gluconate into oxogluconate by *A. xylinum* extract. Reaction mixtures (4 ml.) contained 5 μmoles of substrate (glucose or gluconate), Mickle extract (0·9 mg. of protein N), and phenazine methochloride with and without DPN (100 μg./ml.). The gas phase was air. None of the tested mixtures formed CO_2. Endogenous O_2 uptake (with and without DPN) amounted to 0·7 μmole in 5 hr. Values are plotted after correction for the endogenous values. Copper-reducing power (relative units) in the system oxidizing glucose without added DPN was found to be 100, 4 and <1 respectively at 0, 2 and 3 hr. ▲, ●, Gluconate and glucose without DPN; △, ○, gluconate and glucose with DPN; □, ■, gluconate with and without DPN in the absence of phenazine methochloride.

Renewal of glucose supply in the system after O_2 uptake had ceased resulted in renewal of oxidation. The product of reaction was non-reducing to copper. These observations suggest that the product of glucose oxidation was gluconolactone or gluconate.

Formation of oxogluconates from glucose and gluconate. When the extract with added phenazine methochloride was further supplemented with DPN, CO_2 was again not formed from glucose, but the total O_2 uptake was now twice as high (1·0 mol.-prop.). Moreover, this system oxidized gluconate, taking up 0·5 mol.prop. of O_2 (Fig. 1). It was found that glucose and gluconate had been converted almost quantitatively into oxogluconate (OGn), the yield of 2- and 5-OGn from 5 μmoles of substrate being 4 and 0·6 μmoles respectively. The extract without phenazine methochloride failed to catalyse an anaerobic reduction of DPN by gluconate. Aerobic oxidation of gluconate required the presence of both phenazine methochloride and DPN in the reaction mixture. Thus the observed requirement for DPN need not mean that this substance necessarily intervened in the reaction in the role of an electron carrier.

Formation of carbon dioxide, from glucose in the presence of adenosine triphosphate. The extract with added DPN and phenazine methochloride oxidized glucose at substantially the same rate in the presence and absence of ATP. In the presence of ATP, however, the oxidation proceeded with evolution of CO_2. The initial R.Q. was approx. 0·7, the rate of O_2 uptake being 10 μmoles/hr./mg. of protein N, i.e. of the same order of magnitude as observed in a suspension of whole cells (Fig. 2). The yield of CO_2 attained >4 glucose equivalents/mole of added ATP, i.e. significantly >2. These results suggest that a reaction induced by ATP was one which generates ATP.

Formation of phosphate esters from glucose and gluconate. In the presence of extracts of the cells, glucose and gluconate were phosphorylated by ATP. With extract prepared from cells grown in fructose, fructose was also phosphorylated. Satisfactory equivalence between the decrease in free hexose and that of acid-labile P was observed in experiments in which alumina extract acted anaerobically in the presence of fluoride (Table 1). With Mickle extract in the same conditions, a large discrepancy between the decreases in acid-labile P and free hexose was experienced, the loss of the latter being the greater. An explanation of the discrepancy might perhaps be suggested by the finding of Schramm & Racker (1957) that the *A. xylinum* extract comprises an enzyme system or systems which split F 6-P and xylulose 5-phosphate anaerobically into acetyl phosphate (acetyl-P) and carbohydrate phosphate (erythrose 4-phosphate and triose-P respectively) with attendant uptake of P_i.

The experiment with glucose reported in Table 1 was repeated on a larger scale in an attempt to obtain unequivocal evidence for the presence of glucokinase. The reaction mixture contained 42 μmoles each of ATP and glucose. The decreases in acid-labile P and free hexose in this reaction mixture were equal, amounting each to 26 μmoles. The products of reaction were resolved by barium (LePage, 1949*a*) into a water-insoluble fraction (I) and a water-soluble ethanol-insoluble fraction (II).

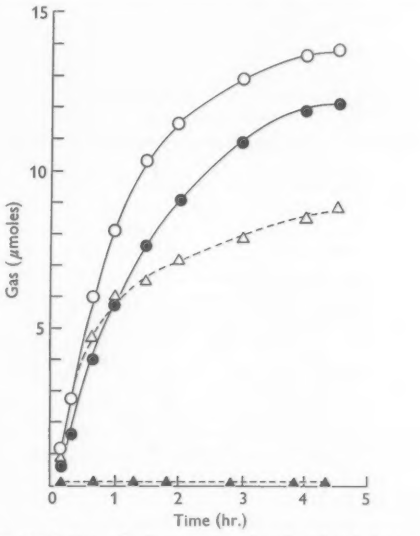

Fig. 2. Oxidation of glucose via phosphorylated intermediates. Reaction mixtures (2 ml.) with or without ATP (300 μg.) contained 0·5 ml. of Mickle extract (0·9 mg. of protein N) of fresh *A. xylinum* cells, 10 μmoles of glucose, DPN (160 μg.), phenazine methochloride and 12 μmoles of $MgCl_2$. The gas phase was air. ○, ●, ΔO_2 and ΔCO_2 with ATP; △, ▲, ΔO_2 and ΔCO_2 without ATP.

Table 1. *Phosphorylation of glucose and gluconate by adenosine triphosphate*

Reaction mixture (2 ml.) with and without substrate (5 μmoles) or ATP (5 μmoles) or both contained 1 ml. of alumina extract (0·7 mg. of protein N), 5 mM-$MgCl_2$ and 10 mM-NaF. Gas phase was N_2. ΔP-ester was calculated from the values of Δ acid-labile P.

System		Changes during 120 min. (μmoles)		
Substrate	ATP	Δ Glucose	Δ Acid-labile P	ΔP-ester
None	+	—	+0·6	—
Glucose	–	−0·2	+0·7	—
Gluconate	–	—	+0·5	—
Gluconate	+	—	−1·7	+2·2
Glucose	+	−2·7	−2·0	+2·7

The amount of G 6-P in II was 11 μmoles (assayed with G 6-P dehydrogenase), and that of F 6-P, 6 μmoles. The total reducing power of (II) proved to be exactly equal to the calculated sum of the reducing powers of these amounts of G 6-P and F 6-P. The sum of recovered hexose-P represents about 60 % of the yield expected on the basis of the observed values of Δ acid-labile P and Δ free glucose. The quotient G 6-P/F 6-P observed is that of a system at equilibrium. Thus the findings suggest that this extract contained phosphohexoisomerase, glucokinase and gluconokinase.

AMP was estimated on the basis of adenine and pentose in fraction (II). These were equal, amounting each to 12·5 μmoles. Assuming myokinase to be present, the observed disappearance of 26 μmoles of acid-labile P would be expected to yield 13 μmoles of AMP. The good correspondence between the observed value and that calculated suggests that myokinase may indeed be present in the extract. α-G 1-P was rapidly equilibrated at pH 7·4 with G 6-P (rate of conversion of G 6-P: $\geqslant 100$ μmoles/hr./mg. of protein N). Thus this system also contained phosphoglucomutase.

As the decreases of free glucose and acid-labile P in the total reaction mixture had been found to be equal, it seemed likely that formed F 6-P had not been further phosphorylated to F 1:6-P. This is also in accord with the finding that fraction (I), which would have contained any formed F 1:6-P, gave a negative test for ketose.

Reduction of pyridine nucleotides by glucose 6-phosphate and 6-phosphogluconate. Both DPN and TPN could be reduced by G 6-P and 6-PG in the cell-free extract. In one set of conditions, G6-P reduced DPN and TPN at roughly equal rates,

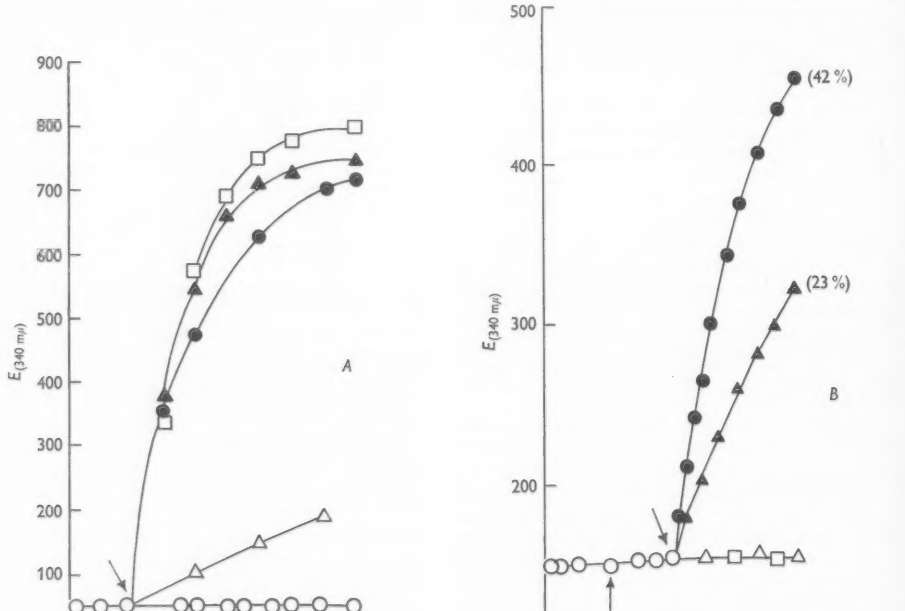

Fig. 3*A*, *B*. G 6-P and 6-PG dehydrogenase activities of *A. xylinum* extract. Reaction mixtures (3 ml.) in systems of Fig. 3*A* contained Mickle extract (0·1 mg. of protein N) of fresh cells, 3 μmoles of substrate (G 6-P or 6-PG), 0·5 μmole of DPN, 0·45 μmole of TPN, 20 μmoles of $MgCl_2$ and 25 mM-cacodylate buffer; pH 6·4. Systems of Fig. 3*B* contained Mickle extract (0·1 mg. of protein N) of frozen cells, 2·5 μmoles of substrate (G 6-P or 6-PG), 0·36 μmole of nucleotide (DPN or TPN), 4 mM-cysteine, 20 mM-NaF and 50 mM-tris buffer; pH 8·2. Light path (quartz cuvette) 1 cm. Values in parentheses beside curves represent the percentage of added nucleotide reduced. ↑, Time of addition of nucleotide; ↓, time of addition of substrate. ○, Systems before substrate addition; ●, 6-PG with DPN; △, 6-PG with TPN; ▲, G 6-P with TPN; □, G 6-P with DPN.

Table 2. *Anaerobic transformation of ribose 5-phosphate into hexose phosphate and heptose phosphate*

Reaction mixtures (3 ml.) with and without R 5-P (12·5 μmoles) contained 1 ml. of Mickle extract (made from 40 mg. dry wt. of fresh cells) with 0·01 M-$MgCl_2$ and 0·01 M-NaF. Gas phase was N_2.

	Composition of reaction mixture after 15 min. (μmoles)				
	Hexose-P	Pentose-P	Heptose-P	P_i	Total
Without added R 5-P	0·8	0·9	0·0	0·8	—
With added R 5-P	6·5	3·7	0·6	1·3*	—
Analytical recovery of added C of R 5-P at 15 min. (%)	55†	26	7	—	88‡

* Despite this observed release of P_i, the amount of non-phosphorylated reducing sugar formed was negligible.
† This value, which corresponds to 34 μatoms of C, was obtained by a direct colorimetric analysis. G 6-P and F 6-P, each determined separately, amounted together to a somewhat larger value, namely 27 + 14 = 41 μatoms of C.
‡ At 90 min. the analytical recovery of added C had fallen to 75%. In a similar system, but without added F^-, the recovery of added C was even lower (50% at 90 min.).

Table 3. *Anaerobic transformation of fructose 1:6-diphosphate into triose phosphate*

Reaction mixtures (2 ml.) with and without F 1:6-P (6·3 μmoles) contained 1 ml. of Mickle extract (made from 20 mg. of dried cells) and neutral 0·06 M-hydrazine sulphate. Gas phase was N_2. Δ Triose-P was calculated from the alkali-labile phosphate.

	Changes during 35 min. (μmoles)		
	Δ Total ketose	P_i	Δ Triose-P
With added F 1:6-P	−4·4	+1·9	+7·5
Without added F 1:6-P	0·0	+0·5	0·0

whereas 6-PG reduced DPN significantly more rapidly than it did TPN (Fig. 3*A*). Under other conditions and with another preparation of extract, TPN but not DPN was reduced by G 6-P whereas DPN but not TPN was reduced by 6-PG (Fig. 3*B*). [Dehydrogenases of G 6-P and 6-PG that are activated both by DPN and TPN have been encountered by other investigators in several bacterial systems. In some of these, the crude mixture of enzymes showed no marked specificity as between DPN and TPN but such specificity became apparent after the enzymes were purified (see, for example, De Moss & Gibbs, 1955).] The reductions of TPN by G 6-P and of DPN by 6-PG were not inhibited by 20 mM-fluoride. The reduction of G 6-P by TPN was not noticeably retarded by 10 mM-monoiodoacetate.

Anaerobic cleavages of carbon chains of carbohydrate phosphates and 6-phosphogluconate. When the Mickle extract of dried cells was incubated in the absence of added nucleotide anaerobically with 6-PG and hydrazine a sluggish production of alkali-labile P plus pyruvate took place (about 0·4 μmole/hr./mg. of protein N). Therefore it seems probable that cleavage of 6-PG to triose-P and pyruvate (Entner & Doudoroff, 1952) did not play a significant role in the metabolism of glucose by this extract. The formation of pyruvate was not augmented by 5 mM-arsenite.

R 5-P was rapidly converted anaerobically into hexose-P (equilibrium mixture of G 6-P and F 6-P) and heptose-P (Table 2). This transformation is consistent with the presence of the following enzymes in the extract: phosphopentoisomerase, phosphopentoepimerase, transketolase, transaldolase and phosphohexoisomerase. Carbon balances drawn up on this basis were found to be incomplete, the deficit increasing as the reaction time increased (see footnote ‡ in Table 2). The anaerobic cleavages described by Schramm & Racker (1957), to which reference has already been made, may have accounted for this disappearance of carbon.

Under anaerobic conditions the extract acting in the presence of hydrazine as added trapping agent formed alkali-labile P (presumably triose-P) from F 1:6-P (Table 3). Whether cleavage of F 1:6-P by aldolase or the anaerobic pathways described by Schramm & Racker (1957) or both accounted for this transformation remains undetermined.

Formation of P_i from carbohydrate phosphates. F 1:6-P was split anaerobically with rapid formation of P_i accompanied by release of a relatively small amount of reducing free sugar. For example, it was found that 12 μmoles of F 1:6-P incubated with Mickle extract (equivalent of 20 mg. dry wt. of frozen cells) in 2 ml. of 20 mM-glycylglycine buffer, pH 7·2, gave 8·1 μmoles of P_i and only 0·5 μmole of reducing free sugar in a reaction time of 120 min. The results suggest that F 1:6-P was hydrolysed largely into a hydrolysis-resistant hexose-P (presumably F 6-P in equilibrium with G 6-P). In the same conditions, α-G 1-P was rapidly converted into reducing acid-stable hexose-P (presumably G 6-P in equilibrium with F 6-P) with little or no release of P_i. When β-G 1-P and UDPG were tested as substrates, the values of acid-labile P and of reducing power released by mild acid hydrolysis remained

practically constant. Presumably, these last substrates were not attacked.

In contrast to the behaviour of the extract, the homogenate of the cells exhibited versatile phosphatase activity. P_i was released rapidly by the homogenate from G 6-P, α-G 1-P, R 5-P and ATP in an anaerobic system in tris buffer at pH 7·5.

Oxidation of triose-P to pyruvate via 3-phosphoglycerate. DPN was rapidly reduced by the cell-free extract acting on F 1:6-P and yeast aldolase (Fig. 4). The velocity of this reaction was increased very markedly by the addition of arsenate to the reaction mixture. The reaction was suppressed by mM-monoiodoacetate. TPN was reduced by this system to some extent, but as such reduction soon ceased it conceivably may have been due to presence of a contaminant (e.g. F 6-P) in the F 1:6-P preparation. In this system TPN but not DPN was reduced by G 6-P (Fig. 3*B*). It could be concluded on the basis of these findings that the extract contained triose-P dehydrogenase.

PGA was converted by the extract into pyruvate and P_i (Table 4). Addition of ADP to the system did not alter the yield of pyruvate though it increased that of P_i. In the system containing PGA plus ADP, fluoride suppressed pyruvate formation completely and lowered the value of P_i released to a level equal to that found in the system containing ADP without PGA. Fluoride did not alter the rate of formation of P_i from ADP. To rationalize these findings the concurrence of the following enzymes in this extract could be assumed: phosphoglyceromutase, a fluoride-sensitive enolase, pyruvate kinase and a fluoride-resistant enzyme system forming P_i from ADP. The inability of added ADP to increase the rate of pyruvate formation might be explained by assuming that a catalytic amount of ADP or its equivalent was present in the enzyme system and could be utilized as a P-acceptor in a cyclic manner, the ADP being converted by reaction with phosphoenolpyruvate into ATP and the latter then back again into ADP. Though pyruvate has been found to be readily oxidized by the whole cells and to be rapidly decarboxylated by them under anaerobic conditions (Schramm *et al.* 1957*b*), it was not oxidized by their extract under the conditions in which PGA was converted into pyruvate.

Evidence has been presented above that when the extract acts in conjunction with ATP to oxidize glucose, ATP is generated. Oxidation of triose-P via PGA into pyruvate and the attendant conversion of P_i into high-energy P could have accounted in part for such ATP synthesis. Additional mechanisms for substrate-level phosphorylation in

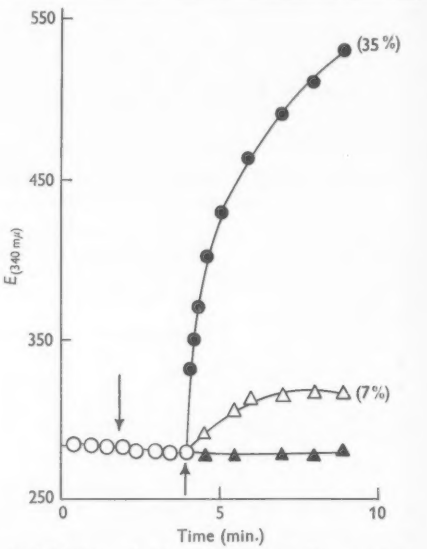

Fig. 4. Oxidation of triose-P by *A. xylinum* extract. Reaction mixtures (2·8 ml.) contained Mickle extract (0·2 mg. of protein N) of frozen cells, crystalline muscle aldolase (50 μg. of protein N), sugar phosphate (2·5 μ-moles), pyridine nucleotide (0·36 μmole), 20 mM-NaF, 17 mM-arsenate (Na^+), 4 mM-cysteine and 50 mM-tris buffer; pH 8·2. Light path (quartz cuvette) 1 cm. Values in parentheses beside curves represent the percentage of added nucleotide found to have been reduced. ↓, Time of addition of pyridine nucleotide; ↑, time of addition of sugar phosphate. ○, Systems before substrate addition; ●, F 1:6-P with DPN; △, F 1:6-P with TPN; ▲, G 6-P with DPN.

Table 4. *Formation of pyruvate from 3-phosphoglycerate*

Reaction mixtures (2 ml.) contained 1 ml. of Mickle extract (15 mg. dry wt. of frozen cells) and 5 mM-$MgCl_2$ in 50 mM-tris buffer, pH 7·8, under N_2. The reaction time was 120 min.

Composition of test system			Products (μmoles)	
PGA (20 μmoles)	ADP (10 μmoles)	Fluoride (12 mM)	Δ Pyruvate	ΔP_i
+	−	−	2·5	3·0
+	+	−	2·5	8·6
+	+	+	0·0	5·2
−	+	−	0·0	5·1

this system have been revealed by the work of Schramm & Racker (1957).

Aerobic formation of carbon dioxide from carbohydrate phosphates. G 6-P was oxidized to CO_2 by a cell-free extract, supplemented only by DPN + TPN, at a slowly falling rate and with an R.Q. slightly less than 1·0. The effect of phenazine methochloride on this system is illustrated in Fig. 5. It is evident that phenazine methochloride increased the velocity of reaction by about fivefold but did not affect the value of R.Q. Conceivably, the phenazine methochloride could have intervened in the oxidation chain as an efficient electron carrier either between reduced pyridine nucleotide and O_2 or between the substrate and pyridine nucleotide, and thus produced the observed increase in reaction rate.

Single addition of either DPN and TPN markedly accelerated the formation of CO_2 from G 6-P (Fig. 6; compare also Fig. 3). However, in these conditions, a mixture of DPN and TPN produced an effect which was significantly greater than the sum of the single effects of the nucleotides.

Oxidations of P-esters to CO_2 were found to be retarded by added adenine nucleotides. For example, 2·5 mM-AMP in an extract supplemented by DPN + TPN + phenazine methochloride lowered the rate of formation of CO_2 from G 6-P by $\geqslant 80\%$. ADP was tested in another experiment in which F 1:6-P was added as substrate and was found to lower the rate of formation of CO_2 by about 50%. The mechanism by which these marked inhibitory effects was accomplished is not apparent to us.

It is noteworthy that in the presence of added Mg^{2+} ions the oxidation of G 6-P attained a high CO_2 yield (83% of theory) (Fig. 7). Reaction in the absence of added Mg^{2+} ions was bimodal in course and showed a steep fall in rate at a relatively low CO_2 yield ($\leqslant 41\%$ of theory) (cf. Fig. 6; see also Fig. 5). In this system, renewal of the G 6-P supply after CO_2 evolution had become slow led promptly to a restoration of a high reaction rate. The findings indicate that the oxidative process occurring in the

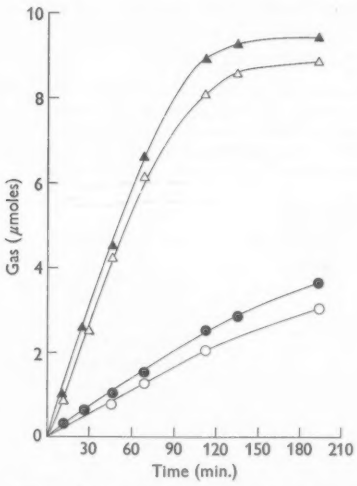

Fig. 5. Effect of phenazine methochloride on the aerobic oxidation of G 6-P by *A. xylinum* extract. Reaction mixtures (2 ml.) contained 5 μmoles of G 6-P, Mickle extract made from 20 mg. of freeze-dried cells, 200 μg. of DPN and 100 μg. of TPN with and without phenazine methochloride. The gas phase was air. The pH was initially about 7 but decreased during the reaction. Values for CO_2 were calculated from the pressure readings on the basis of the assumption that the pH was 6. ○, ●, ΔCO_2 and ΔO_2 without phenazine methochloride; △, ▲, ΔCO_2 and ΔO_2 with phenazine methochloride.

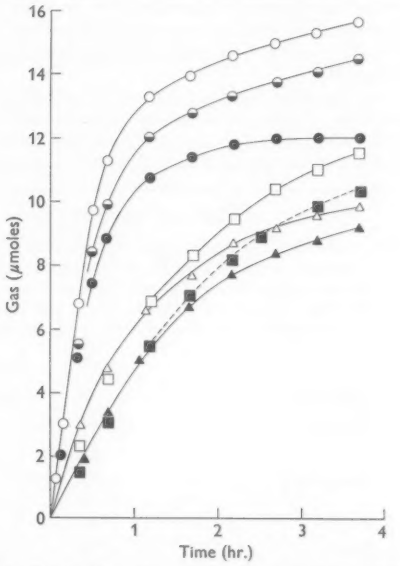

Fig. 6. Effects of pyridine nucleotide on aerobic oxidation of G 6-P by *A. xylinum* extract. Reaction mixtures (2 ml.) with 200 μg. of DPN or 200 μg. of TPN or 200 μg. of their mixture (1:1) and with or without 10 mM-fluoride contained Mickle extract (1 mg. of protein N) of fresh cells, 50 μg. of phenazine methochloride/ml. and 5 μmoles of G 6-P. The gas phase was air. In the system with DPN + TPN, ΔF 1:6-P (μmoles) at 240 min. was found to be 0·5 and 1·0, without and with fluoride respectively. Corresponding values for ΔP_i were 2·0 and 0·5 μmoles. For the method of calculation of CO_2 see the legend to Fig. 5. □, ■, ΔO_2 and ΔCO_2 with DPN; △, ▲, ΔO_2 and ΔCO_2 with TPN; ○, ΔO_2 with DPN + TPN (with and without fluoride); ◒, ●, ΔCO_2 with DPN + TPN without and with fluoride respectively.

absence of Mg^{2+} ions had resulted in an intermediary piling up of a slowly oxidizable product or products. The nature of the latter is still unknown.

The rates of formation of CO_2 from G 6-P, 6-PG and R 5-P in a system consisting of cell-free extract supplemented by DPN + phenazine methochloride were found to be all practically equal. In accord with the assumption that the formation of CO_2 had involved the pentose cycle, 10 mM-fluoride did not decrease the initial rate of these oxidations. Moreover, an extract with added phenazine methochloride + DPN + TPN + Mg^{2+} ions oxidized F 1:6-P to CO_2 at the same rate as it oxidized G 6-P. In both cases the R.Q. was 1·0 (Fig. 7).

Rates of measured part reactions and those of CO_2 formation are collected in Table 5. It can be seen that the part reactions proceeded at rates greater than or in the same rough order of magnitude as the rates of the production of CO_2 from carbohydrate phosphates. Thus the interpretation that the production of CO_2 occurred via observed part reactions does not present difficulty on kinetic grounds.

Homogenates prepared either by grinding the cells with alumina or exposing them to vibration with glass beads as described in the Methods section, sediments prepared from such homogenates

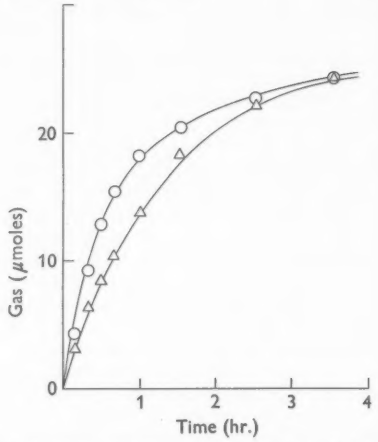

Fig. 7. Aerobic oxidation of F 1:6-P and 6-P by *A. xylinum* extract. Reaction mixtures (2 ml.) contained 1 ml. of Mickle extract (made from 20 mg. dry wt. of frozen cells), 5 μmoles of substrate (G 6-P or F 1:6-P), 200 μg. of TPN, 200 μg. of DPN, 100 μg. of phenazine methochloride and 4 mM-$MgCl_2$. The gas phase was air. The R.Q. of all the oxidations was 1·0 throughout, both with G 6-P and F 1:6-P. ○, G 6-P; △, F 1:6-P.

Table 5. *Reactions catalysed by a cell-free extract of* A. xylinum

Cell-free Mickle extracts of glucose-grown cells were used. Substrate concentration varied from 2 to 5 μmoles/ml. Systems were buffered with tris at pH 7·4 and 8·2, and with citrate (Na^+) at pH 6·4. The temperature was 30°. Aerobic systems were supplemented with phenazine methochloride (100 μg./ml.) as added electron carrier. To anaerobic systems 10 mM-fluoride was added in order to inhibit side reactions catalysed by phosphatases. Mixtures tested at pH 8·2 contained 4 mM-cysteine. Gas phase was air or N_2. Triose-P was generated from F 1:6-P by crystalline aldolase.

Substrate	O_2	pH	Measured products and their rates of formation (μmoles/hr./mg. of protein N)	Cofactors*
Glucose	+	6·4	CO_2, 0·0; Gluconate, 4	—
	+	7·5	CO_2, 4	ATP, DPN, Mg^{2+} ions
	−†	6·4	G 6-P, 3	ATP, Mg^{2+} ions
Gluconate	+	6·4	CO_2, 0·0; 2-OGn, 2	DPN
	−†	6·4	6-PG, 3	ATP, Mg^{2+} ions
F 6-P	−†	6·4	F 1:6-P, 0·0	ATP, Mg^{2+} ions
G 6-P	+	7·4	CO_2, 12	DPN, TPN
	+	7·4	CO_2, 5	DPN
	−	7·4	F 6-P, ⩾50	—
	−	8·2	TPNH, 15	TPN‡
α-G 1-P	−	7·4	G 6-P, ⩾100	—
F 1:6-P§	−	7·4	Triose-P, 8	—
R 5-P	+	7·4	CO_2, 5	DPN
	−	7·4	F 6-P, 10	—
6-PG	+	7·4	CO_2, 5	DPN
	−	8·2	DPNH, 30	DPN‡
Triose-P	−	8·2	DPNH, 20	DPN,‖ AsO_4^{3-} ions
PGA	−	7·4	Pyruvate, 3	—

* Components listed are those which markedly accelerated reaction.
† Extract was prepared by grinding cells with alumina.
‡ Under another set of experimental conditions, both DPN and TPN were able to accelerate this reaction.
§ Hydrazine was added as trapping agent. Fluoride was not added.
‖ TPN was not reduced.

by centrifuging and supernatant fluids separated by the same means failed to form cellulose from glucose, G 6-P, UDPG, α-G 1-P, β-G 1-P and a crude specimen of G 4-P in the presence of a range of tested cofactors. As the outcome of these experiments was negative they are not described in detail. G 6-P incubated aerobically in a suspension of whole cells within their Mickle extract supplemented by a mixture of cofactors gave no cellulose. This result suggests that during the oxidation of G 6-P by the cell extract no cellulose precursor which could be polymerized by the cells on their outer surface had been formed.

DISCUSSION

A scheme of alternate pathways for glucose metabolism

In constructing a scheme for glucose metabolism in this organism, it is necessary to make provision for the following observations (Schramm *et al.* 1957*a*, *b*). (1) The isotope configuration of cellulose formed by the cells from specifically-labelled [^{14}C]hexoses corresponds to that of hexose-P in a pentose cycle. (2) The cells form cellulose not only from glucose but also from gluconates (gluconate, 2-OGn, 5-OGn) and certain three-carbon substrates (glycerol and dihydroxyacetone), and, after adaptation, from fructose. (3) Cells acting on glucose rapidly form gluconate, demonstrable in the medium. Cells injured selectively by freeze-drying or by 2:4-dinitrophenol convert glucose into 2- and 5-OGn, which pile up in the medium. (4) Depending on experimental conditions and the history of the cells, 2- and 5-OGn may be converted into carbon dioxide with and without attendant formation of cellulose. (5) Pyruvate, acetate and citrate-cycle intermediates, though oxidized by the cells to carbon dioxide, do not yield cellulose. (6) The concentration of fluoroacetate which stops acetate oxidation does not block cellulose formation from glucose. (7) A concentration of fluoride which

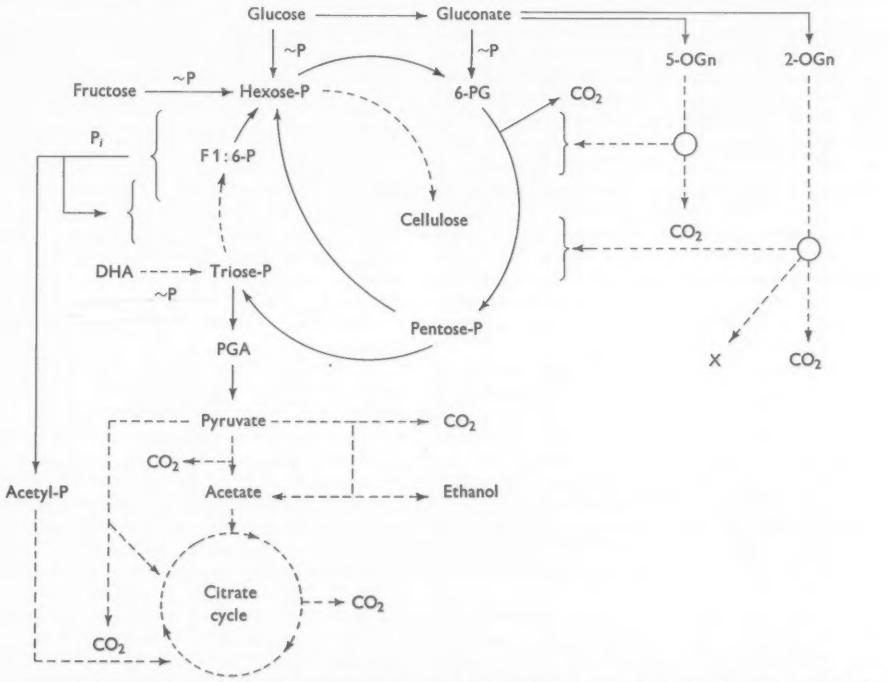

Fig. 8. Scheme of alternate pathways of oxidative carbohydrate metabolism in *A. xylinum*. Reactions drawn with a continuous line have been demonstrated in the cell-free extract. Reactions not so demonstrated but which probably occur in the intact cell are drawn with a broken line. ~P indicates an uptake of ATP. Anaerobic cleavages by which pentose-cycle intermediates are converted into acetyl-P with uptake of P_i are represented by an arrow on the left-hand side of the scheme. Pathways of 2- and 5-OGn metabolism whose mode of entry into the pentose cycle and ways of by-passing it are not known are represented by arrows on the right-hand side of the scheme. An unknown product of 2-OGn metabolism has been designated *X*.

blocks *A. xylinum* enolase in an extract does not interfere with the production of carbon dioxide from glucose by a suspension of cells.

In addition to the above observations made on whole cells, the scheme of metabolism to be constructed should also take account of those reactions pertinent to carbohydrate oxidation that have been demonstrated by us in cell-free preparations of the organism, namely the following: (1) oxidation of glucose via gluconate to OGn (largely 2-OGn); (2) phosphorylation of glucose, gluconate and fructose; (3) oxidation of phosphorylated carbohydrates and 6-PG via a pentose cycle; (4) oxidation of triose-P via PGA to pyruvate; (5) oxidation of tri- and di-carboxylic acids to carbon dioxide (unpublished experiments); and (6) anaerobic cleavages of F 6-P and xylulose 5-phosphate leading to formation of acetyl-P with uptake of P_i (Schramm & Racker, 1957) in a manner resembling a cleavage of pentose-P which was first described by Heath, Hurwitz & Horecker (1956).

It is evident that no single known pathway of glucose metabolism can account by itself for the versatile activities of *A. xylinum* cells and their extracts. A scheme which presents alternate pathways for glucose metabolism is therefore proposed (Fig. 8). It is predicated upon the notion that these cells operate a pentose cycle and that the intermediate of the cycle which is closest to cellulose is a hexose-P. Thus substrates which give cellulose are all shown as being capable of entering the pentose cycle, whereas substrates which give carbon dioxide without attendant formation of cellulose are shown by-passing the pentose cycle. With 2- and 5-OGn, pathways which enter the cycle and others which by-pass it are indicated [in this connexion, cf. Wood (1955) and De Ley (1954)].

The pentose cycle shown is one entered in different ways: the way mediated by glucokinase and several other ways in which primary conversion of glucose into gluconate plays a part. It is indicated that gluconate can be introduced into the cycle directly by the action of gluconokinase and indirectly via 2- and 5-OGn respectively.

Several ways by which carbon can be withdrawn from the pentose cycle and fed into a citrate cycle are shown. One of these is an oxidative shunt operated at the level of triose-P, similar to that which has been found in conjunction with the pentose cycle in a number of other micro-organisms (see, for example, Vandemark & Fukui, 1956). Additional means by which carbon can be moved out of the pentose cycle and fed into the citrate cycle involve anaerobic transformations of pentose-cycle intermediates leading to formation of acetyl-P.

The pentose cycle is thought to be a major pathway of nucleotide syntheses and as such might be essential for cell proliferation. The failure of some strains of *A. xylinum* to grow on acetate (or acetate-forming ethanol) as a sole carbon source in a medium containing ammonium as a nitrogen source (Shimwell, 1957*a*), whereas they grow on glucose in this medium and can utilize acetate in the presence of glucose, is consistent with the proposal that the pentose cycle can be entered in such strains by glucose but not by acetate and that the pentose cycle plays an essential role in growth.

Among the enzymes that are concerned with glycolysis via the classical Embden–Meyerhof pathway, phosphofructokinase was conspicuous by its apparent absence from an extract of *A. xylinum*. The finding that these cells did not metabolize glucose anaerobically was consistent with this result. The fact that anaerobic oxidations of glucose by dye systems and ferricyanide did not sustain either cellulose synthesis or formation of carbon dioxide by the cell suspension (Schramm *et al.* 1957*b*) can probably be traced to the reason that such oxidations do not generate ATP. It is not as easy, however, to rationalize the finding that these cells possess anaerobic mechanisms for the cleavage of carbohydrate phosphate esters with attendant uptake of P_i and generation of high-energy P yet fail to form cellulose anaerobically from glucose.

Note on biochemical evolution in Acetobacter

It is evident that a series of successive intermediary steps of carbohydrate oxidation in *A. suboxydans* (cf. Hauge, King & Cheldelin, 1955; Kitos, King & Cheldelin, 1956; Fewster, 1956) and in *A. xylinum* are identical. It should also be noted, however, that there are at least two enzyme functions in *A. xylinum* which appear to be missing from *A. suboxydans*, namely, (1) an ability to convert a P-ester participating in the pentose cycle into cellulose, and (2) an ability to oxidize acetate to carbon dioxide. Both functions are positioned terminally in the metabolic scheme; neither of them need be regarded as indispensable to the metabolic utilization of carbohydrates by *A. xylinum*, either for energy or carbon. It has indeed been demonstrated experimentally that in *A. xylinum* the mutative deletion of the property of cellulose production is compatible with survival of the cell and with vigorous proliferation (Schramm & Hestrin, 1954). This loss mutation, moreover, is a reversible one (Shimwell, 1956) and consonant with further mutations by which strains indistinguishable from established type species of *Acetobacter* arise under laboratory conditions from an *A. xylinum* parent (Shimwell, 1957*b*). As yet, mutative loss or acquisition of the ability to oxidize acetate does not appear to have been encountered experimentally in this bacterial genus. Still, the notion that this ability is likewise a mutable property seems permissible. Biochemically, the

hypothesis that *A. xylinum* is a phylogenetic ancestor of *A. suboxydans* thus might imply only that *A. xylinum* has been able to undergo consecutive loss mutations of two non-essential enzyme systems. An opposing view, namely that evolution proceeded from a simple state to a more complex one, may be more attractive on philosophical grounds. In that case, the interesting possibility has to be considered that the ancestor of *Acetobacter* was equipped with a mutative potential so broad as to comprise within its range two enzyme systems (respectively for cellulose synthesis and acetate oxidation) which are facultative in *Acetobacter* but have elsewhere assumed cardinal roles in cellular survival.

SUMMARY

1. A cell-free extract of *Acetobacter xylinum* failed to form cellulose from a range of tested substrates.

2. The extract (with added phenazine methochloride) oxidized glucose aerobically to gluconate. When this system was further supplemented with diphosphopyridine nucleotide, it oxidized gluconate to oxogluconate (largely 2-oxogluconate).

3. The extract with adenosine triphosphate oxidized glucose aerobically to carbon dioxide. The extract contained kinases (glucokinase and gluconokinase), phosphoglucomutase, a complete pentose-cycle set of enzymes, and a set of enzymes by which triose phosphate could be converted into pyruvate.

4. A scheme of alternate pathways for glucose metabolism that can be operated in an *A. xylinum* cell is proposed. The scheme shows a pentose cycle which has several ports of entry and exit. The latter lead from the pentose cycle into a citrate cycle.

5. Biochemical evolution in the genus *Acetobacter* is briefly discussed.

Note added in proof. See Colvin, J. R. (1957). *Arch. Biochem. Biophys.* **70**, 294. Glaser, L. (1957). *Biochim. biophys. Acta*, **75**, 436. Greathouse, G. A. (1957). *J. Amer. chem. Soc.* **79**, 4503.

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The Excretion of *allo*Tetrahydrocortisol in Human Urine

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It is now agreed that cortisol (11β:17α:21-trihydroxypregn-4-ene-3:20-dione; hydrocortisone) is the principal glucocorticoid hormone secreted by the normal human adrenal cortex (Mason & Sprague, 1948; Bush & Sandberg, 1953; Romanoff, Hudson & Pincus, 1953; Sweat, Abbott, Jeffries & Bliss, 1953; Roberts & Szego, 1955). As with other steroids having the Δ^4-3-keto group in ring A, the principal known metabolites of cortisol are steroids in which this group has been reduced to a saturated 3-hydroxyl group (Fig. 1) (Lieberman & Teich, 1953; Fukushima *et al.* 1955). Although four possible stereoisomeric 3-alcohols exist, only one has been found so far among the C_{21} metabolites of cortisol

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(and cortisone), namely that having the configuration 3α-OH-5β(H). On the other hand, all but one of the known saturated 3-alcohols found in adrenal-gland extracts have the configuration 3β-OH-5α(H) (Reichstein & Shoppee, 1943). Klyne (1954) has pointed out that most of the natural steroids of this type fall into these two isomeric classes, which are the two in which the 3-hydroxyl group is in the equatorial, and thermodynamically more stable, configuration. By contrast, C_{21} steroids lacking a 17-hydroxyl group (Dorfman, 1954*a*; Dorfman & Ungar, 1953), and C_{19} steroids, when containing a Δ^4-3-keto group, are reduced to a mixture of isomers in which both 3α-OH-5α(H) and 3α-OH-5β(H) configurations make up appreciable proportions of the mixture (Dorfman, 1954*a*; Lieberman & Teich, 1953; Roberts & Szego, 1955). The relative proportions of such isomers have become of great interest and of some practical importance, since Dorfman (1954*a*, *b*, 1955) showed that they were determined mainly by substituents in other parts of the steroid nucleus. Thus it is possible on Dorfman's theory to calculate the relative secretion rates of the different steroid hormones having a Δ^4-3-keto group in man by estimating the appropriate isomers among their known urinary metabolites. In addition Dorfman (1954*a*) has used some of his findings to suggest that the reactions involved in the metabolism of cortisol to C_{19} metabolites *in vivo* take place in a particular sequence. The theory, however, depends much upon the inability of previous workers to demonstrate any appreciable quantities of a 3α-OH-5α(H) steroid among the major metabolites of cortisone or cortisol (e.g. Burstein, Savard & Dorfman, 1953).

In an examination of the reducing steroids of several hundred urine samples by an extension of methods described earlier (Bush, 1952, 1954; de Courcy, Bush, Gray & Lunnon, 1953) a reducing substance, which had an R_F slightly greater than that of tetrahydrocortisol (3α:11β:17α:21-tetrahydroxy-5β-pregnan-20-one) and which was not present in the urine of patients with adrenal insufficiency (Bush & Lovell, unpublished work; Bush & Willoughby, 1957), was noticed in nearly all of the samples. In this paper evidence will be given that this substance is *allo*tetrahydrocortisol (3α:11β:17α:21-tetrahydroxy-5α-pregnan-20-one) and that it is a normal metabolite of cortisol and cortisone in man. This substance has long been known as a component of adrenal-gland extracts (Wintersteiner & Pfiffner, 1935; Reichstein, 1936; Mason, 1936, 1938; Kuizenga, 1939) and recently has been shown to be a metabolite of cortisol in isolated perfused rat livers (Caspi, Levy & Hechter, 1953; Caspi & Hechter, 1954). It is peculiar in being one of the few natural C_{21} steroids of animal origin having the axial configuration of its 3-hydroxyl group.

EXPERIMENTAL

Materials

Methanol. This was heated under reflux for 1 hr. with 25 g./l. of KOH and 20 g./l. of granulated zinc. After being filtered, it was distilled slowly through a 25 cm. fractionating column. It was stored in brown foil-stoppered bottles.

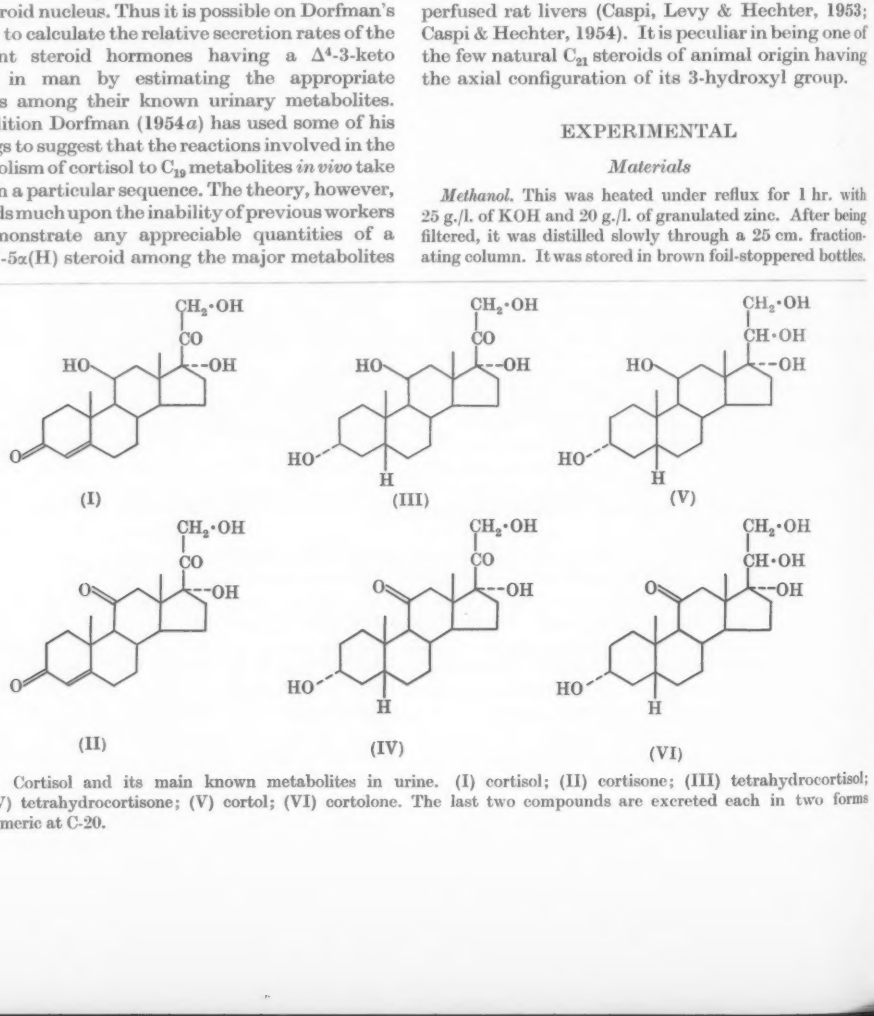

Fig. 1. Cortisol and its main known metabolites in urine. (I) cortisol; (II) cortisone; (III) tetrahydrocortisol; (IV) tetrahydrocortisone; (V) cortol; (VI) cortolone. The last two compounds are excreted each in two forms isomeric at C-20.

Ethanol. This was heated under reflux for 2 hr. with 5 g. of 2:4-dinitrophenylhydrazine and 10 ml. of conc. HCl/l. It was then cooled, filtered and distilled through the 25 cm. column. It was redistilled through the same column at 200 ml./hr.

Benzene. Benzene (750–1500 ml.) was filtered slowly through 100 g. of silica gel (chromatographic grade, Hopkin and Williams Ltd.) holding 20 ml. of conc. H_2SO_4 on a sintered-glass funnel (porosity 2, sintered surface 12 cm. in diameter). After being washed with water and dried over $CaSO_4$ it was distilled slowly through the 25 cm. column and stored in brown foil-stoppered bottles.

Light petroleum (b.p. 100–120°). This was purified in the same way as benzene.

Ethyl acetate. This was washed with 0·25 vol. of 10% Na_2CO_3 and 0·25 vol. of water immediately before use. The wet solvent was used for extracting aqueous phases; for extracting or transferring solid residues the washed solvent was dried over Na_2SO_4 immediately before use.

Ether. This was washed immediately before use with 0·25 vol. of saturated aqueous ferrous sulphate and 0·25 vol. of water. It was dried or used wet as for ethyl acetate.

Pyridine (*A.R.*). This was stored in dark bottles over barium oxide.

m-*Dinitrobenzene.* This was purified by sublimation at atmospheric pressure.

Blue tetrazolium chloride [3:3′-dianisolebis-4:4′-(3:5-diphenyl)tetrazolium chloride]. No satisfactory sample could be obtained from commercial houses, so the reagent was prepared by the method of Rutenburg, Gofstein & Seligman (1950). In the early part of the work an excellent sample, kindly supplied by Dr J. Walker (National Institute for Medical Research), was used.

Sodium bismuthate. Before use each batch was tested by oxidizing 50–100 μg. of cortisol and examining the products chromatographically. Only those batches giving full yields of 11β-hydroxyandrost-4-ene-3:17-dione were used.

Ethanolic potassium hydroxide. A.R. KOH was dissolved in purified ethanol and titrated with HCl. It was then adjusted to 3N and used at once. In the early part of the work pellets were used, but much better results have been obtained recently by using the stick form. Poor results seemed to be due to the frequent presence of brown-stained pellets (iron?) in so-called A.R. grades; sticks with brown stains were less frequent and much easier to detect; batches with stained sticks were rejected.

Blue tetrazolium reagent. Freshly purified blue tetrazolium chloride was made up in purified ethanol as a 1% (w/v) solution and kept in the refrigerator. The stock solution was stable for at least 3 months. The reagent was prepared immediately before use by mixing 2 vol. of stock solution with 5 vol. of water and adding 3 vol. of 4N-NaOH. The reagent was stable enough for use up to 0·5 hr. after making.

Glucuronidase and sulphatase preparations. Roman snails (*Helix pomatia*) were obtained from Watkin and Doncaster (Welling, Kent) and fed on filter paper in a warm room until they were lively. They were then drowned for 15–18 hr. in a closed bucket. The shells were clipped away with strong scissors and an incision 2–3 cm. long made in the back, starting behind the head. The alimentary canal was then allowed to hang from the incision over a small conical flask and snipped open, when the viscous brown succus entericus drained into the flask. The juice was collected from a hundred snails before being mixed and assayed for glucuronidase by the method of Talalay, Fishman & Huggins (1946). It usually contained 60,000–90,000 Fishman units/ml., each snail yielding about 0·7 ml. The mixed enzyme preparation lost only 20% of its activity after being stored in the refrigerator for 7 months. The mixed preparation did not metabolize or destroy added steroids of the type concerned in this investigation and we did not find it necessary to purify our preparation (cf. Henry & Thevenet, 1952). Sulphatases were not assayed, but it was shown in tests on pure dehydro*epi*androsterone (DHA) sulphate that complete hydrolysis of this substance occurred in sodium acetate buffer under our conditions. With urine, hydrolysis of DHA was not complete, but the inhibition was less severe than was expected from the results of Dodgson & Spencer (1953) and was not increased by raising the phosphate concentration. The yield of dehydro*epi*androsterone from its sulphate in urine was 55% by the method described below, and did not differ significantly with different samples of urine.

Filter paper for chromatography. Whatman no. 3 MM and no. 2 were used either as strips or sheets. Before use the strips (22½ in. × 1 in.) of no. 2 and of no. 3 MM (22½ in. × 2 in.) were washed chromatographically (Isherwood & Hanes, 1953) in batches of 100–200 by using successively ethanol–aq. NH_3 soln. (sp.gr. 0·88) (1:1, v/v), ethanol and ether–ethanol (1:1, v/v). After being dried in the fume cupboard they were stored in the dark, wrapped in aluminium foil. When used for isolating material for ultraviolet spectroscopy, the strips were given an additional wash shortly before use by extraction under reflux in a Soxhlet apparatus with benzene–methanol (1:1, v/v).

METHODS

Medical. Patients under treatment with adrenocorticotrophic hormone (ACTH) were given 25 i.u. of ACTHAR gel (Armour) intramuscularly, at 6.0 a.m. and 6.0 p.m. Cortisone was given orally in tablets usually 3–4 times daily; doses ranged from 25 to 200 mg./day. Normal subjects included staff, and patients not seriously ill and not suffering from endocrine disease. Other subjects were patients suffering from various degrees of hirsutism, or mild to moderate Cushing's syndrome.

Urine collection. Samples were collected between 6.0 a.m. and 6.0 a.m. in 2·5 l. bottles containing 100–150 ml. of toluene. The urine was passed into the bottle or transferred to it immediately after each specimen had been passed. The 24 hr. samples were transferred to the cold room (2–4°) within 6 hr. of completion.

Hydrolysis of steroid conjugates. A known fraction (usually 0·05 or 0·2 of a 24 hr. sample) of each urine sample was heated on a boiling-water bath for 20 min. in a conical flask, after it had first been checked that the pH lay between 5·0 and 6·5. The sample was then cooled in tap water and made up to the original volume. 5M-Acetate buffer (0·1 vol.) at pH 4·8 was added and the pH of the sample brought to 4·8 by adding 0·5M-acetic acid and sodium acetate. Succus entericus of *Helix pomatia* (0·02 vol.) was added (Henry & Thevenet, 1952; Jarrige & Henry, 1952). After mixing, the sample was incubated at 45° for 16–18 hr. Tests in which urine samples were incubated, extracted and then buffered and reincubated with fresh enzyme solution showed that

glucuronosides of the principal known steroid metabolites (C_{21} and C_{19}) were completely hydrolysed by this method. Sulphates were only partly hydrolysed (see above) but the yield of DHA from its sulphate was surprisingly constant at 50–60% (i.e. yield over whole procedure including extraction, washing and chromatography). This recovery figure was obtained both by adding pure DHA sulphate to urine samples and by comparing the yield of samples treated enzymically with the yield from portions of the same samples treated by the method of Paterson & Swale (1953).

Extraction. The urine was extracted, after incubation, in cylindrical separating funnels twice with 3 vol. of ether–ethyl acetate (2:1, v/v). The combined extracts were washed three times with 0·05 vol. of N-NaOH and once with 0·05 vol. of water. Two drops of acetic acid were added and the extract was partially dried by filtering slowly through approx. 5 g. of anhydrous Na_2SO_4. It was then distilled to approx. 0·5 ml. on a water bath at 45° by using a fine capillary leak and a high-capacity condenser with a Hivac pump. The extract was then transferred with ethyl acetate and methanol to a 15 ml. conical centrifuge tube. This solution was evaporated to dryness with a fine jet of air (filtered through pellets of sodium hydroxide and glass wool), with the tube immersed in water at 30–40°. No artifacts or serious losses were noticed with this method as long as the air was filtered, the solvents were purified as described and the extracts well washed.

Chromatography. The general procedure was as described by Bush (1952), but most of the work was done at room temperature with the slightly modified systems of Bush (1954). Solvent systems used were: (1) benzene–methanol–water (2:1:1, by vol.); (2) light petroleum–benzene–methanol–water (66:33:80:20, by vol.); (3) light petroleum–methanol–water (25:24:1, by vol.).

Nearly all the material used in this work was isolated from fractions used for the estimation of several classes of steroids. The urine extracts were therefore subjected to a preliminary chromatogram on strips of Whatman no. 3 MM paper 22½ in. × 2 in. by a single-length run in system 2. The strips were then cut into three parts and eluted chromatographically with ethyl acetate–methanol (2:1, v/v). The most polar fraction (fraction 3, Fig. 2) was used for the present study. The details of the chromatography and reactions used to identify *allo*tetrahydrocortisol are described below.

Quantitative estimations. Fraction 3 was evaporated to dryness with a filtered air jet after elution from the preliminary chromatogram on Whatman no. 3 MM paper, and dissolved in 1 ml. of ethanol in a glass-stoppered test tube (3 ml. capacity). A volume equivalent to 0·01 of a 24 hr. urine sample was deposited over the whole width of a 2·5 cm. wide strip of Whatman no. 2 paper in a zone about 4·5 cm. long. It was then concentrated by running up with ethyl acetate–methanol (2:1, v/v), to the starting line (Bush, 1952). After equilibration for 3 hr. in system 1 such strips were run for 15–18 hr. and then dried in the fume cupboard for 0·5 hr. or more. The dry strip was then passed twice over blue tetrazolium reagent in a 10–20 cm. diameter watch glass so that it was wetted from the underside only. Each passage over the surface of the reagent took 20 sec. for a 40 cm. strip of paper and was made in the same direction (i.e. not a pendular movement) at a regular speed. The wet strips were then hung horizontally for 20 min. in still air. This way of dipping and hanging the strips was essential to obtain a constant blank value when the strip was scanned in the absorptiometer. The damp strips were then hung vertically in a tank into which was passed a steady stream of SO_2 from a siphon. The concentration of SO_2 was not critical as long as it was sufficient to cause a spot of B.D.H Universal Indicator at the top of the strip to turn red within 1–2 sec. of putting the strip in the tank. After 20 min. in SO_2 the strips were removed and dried in air for at least 2 hr. The dry strips were then mounted in the enlarged carrier of an EEL Electrophoretic Scanner (Evans Electroselenium Ltd., Essex) specially modified to take 45 cm. strips. The cell was screened with a card diaphragm having a slit 8 mm. horizontally × 22 mm. vertically (i.e. with 1·5 mm. cut off each edge of the strip), and a sheet of Ilford Microfilter no. 4 (Minus Blue). This filter transmits 5300 Å to the infrared. Transmission falls off smoothly with decreasing

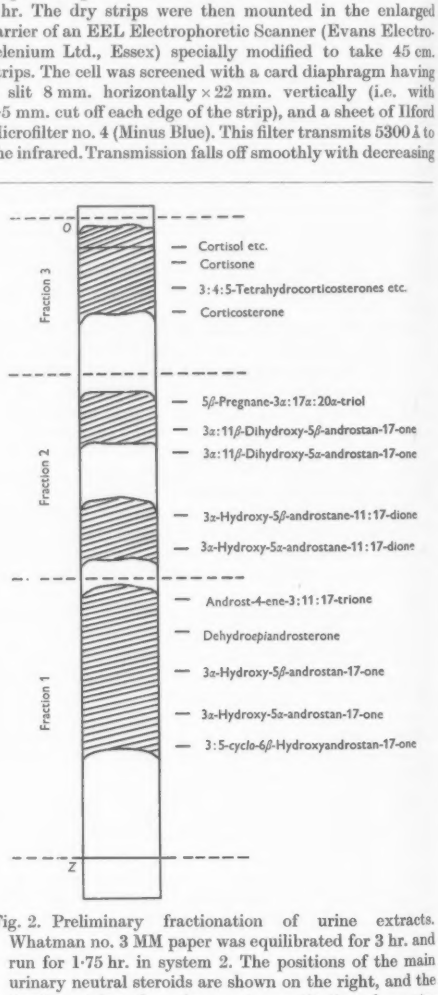

Fig. 2. Preliminary fractionation of urine extracts. Whatman no. 3 MM paper was equilibrated for 3 hr. and run for 1·75 hr. in system 2. The positions of the main urinary neutral steroids are shown on the right, and the fractions taken for subsequent quantitative chromatography on the left. The paper is cut at the dotted lines for eluting the fractions. *O*, origin; *Z*, solvent front; *OZ* measures 40 cm. at 21–24°. The shaded zones are those obtained when a typical urine extract is run and examined with alkaline *m*-dinitrobenzene (fractions 1 and 2) and blue tetrazolium reagent (fraction 3).

wavelength to a value of 0·1% at 5000 Å. Transmission is negligible in the ultraviolet range. The strips were then 'scanned' by hand at intervals of 1–2 mm. The curves obtained were then compared with the strips, the peaks marked off, and a base line was ruled for each peak, by using a fixed convention for all strips. Overlapping peaks were 'split' into their components by careful reconstruction, the profiles from chromatograms of pure steroids and subtraction being used to obtain the profile of the adjacent compound. Chromatograms run for 15–18 hr. were sufficient to give complete separation, by inspection, of the compounds considered here, but slight overlap of the profiles was usually found on scanning. Measurements were not considered reliable if overlap made reconstruction difficult; this, however, was not common unless the room temperature varied greatly during chromatography or some other obvious fault occurred. The areas of each (reconstructed) peak were then measured with a planimeter, and the quantities read off from a calibration curve, constructed similarly from chromatograms of known quantities of pure steroids. A calibration curve was made with four or more quantities of appropriate standards for each batch of estimations. The error of this method has not yet been examined statistically, but during the period of this study, with four-point calibration curves over the range 5·0–80 μg., the great majority of points lay within $\pm 5\%$ of the mean linear regression of quantity on optical density, and no points lay more than $\pm 10\%$ from this line.

A fuller account of this method, its sources of error and its application to other types of steroid is in preparation. Richardson, Touchstone & Dohan (1955) have described a similar method for reducing steroids by means of an adaptor on the Beckman Spectrophotometer.

Methods of detection on chromatograms. Reducing steroids were detected with blue tetrazolium reagent as above, the second dip and treatment with SO_2 being omitted. Δ^4-3-Oxo steroids were detected by the NaOH fluorescence test (Bush, 1952) with 2·7 N-NaOH containing 0·2 ml. of 0·5% (w/v) ethanolic blue tetrazolium chloride/90 ml. of NaOH. 17-Oxo steroids were detected by dipping the chromatograms over the surface of a reagent, made up immediately before use by adding 1 vol. of ethanolic 3 N-KOH to 2 vol. of freshly made 2% (w/v) *m*-dinitrobenzene in ethanol. The wet strip was then dried for 10–20 sec. in the draught of a fume cupboard and finally exposed for 80 sec. to a battery of five electric-fire elements, at a distance such that an ordinary mercury thermometer read 45–47°. Steroids with a glycerol or 17:20-diol side-chain were detected by the method of Bush (1955).

Acetylation. Dry residues containing 20–50 μg. of steroid were dissolved in 2 drops of acetic anhydride and 4 drops of pyridine in a stoppered tube 10 mm. × 12 mm. and left in the dark in a desiccator. The material was then dissolved in ethyl acetate and washed with small volumes of N-Na_2CO_3, N-HCl and water. The washed solution was filtered slowly through 0·5 g. of anhydrous Na_2SO_4 and evaporated with an air jet. The residue was dissolved in ethanol for deposition on chromatograms.

Oxidation with sodium bismuthate (Norymberski, 1952, 1955). The dried material was dissolved in 0·5 ml. of 50% acetic acid and 25 mg. of $NaBiO_3$ added. The suspension was shaken mechanically for 1 hr. in a 100 mm. × 12 mm. stoppered tube screened with black paper. Water (5 ml.) and 10 ml. of saturated $NaHCO_3$ were added and the mixture was extracted twice with 45 ml. of ethyl acetate–ether (1:2, v/v). This extract was filtered slowly through 1 g. of anhydrous Na_2SO_4 and evaporated under reduced pressure on a water bath at 45°.

Oxidation with chromic acid. (Lieberman, Katzenellenbogen, Schneider, Studer & Dobriner, 1953). 17-Oxo steroids and their esters were dissolved in 0·3 ml. of acetic acid and 0·2 ml. of 2% (w/v) aqueous CrO_3 was added. After mixing, the tube (100 mm. × 12 mm.) was stoppered and left at room temperature in the dark for 15–18 hr. It was then diluted with 2·0 ml. of water and extracted with 3 ml. of ethyl acetate. The extract was washed twice with 1 ml. of saturated $NaHCO_3$ and once with 1 ml. of water. After being filtered slowly through anhydrous Na_2SO_4 the extract was evaporated with an air jet or under reduced pressure and dissolved in ethanol for deposition on chromatograms.

21-Acetoxy steroids were dissolved in 0·2 ml. of acetic acid and 0·1 ml. of 1·5% aqueous CrO_3 was added. The stoppered tube was left in the dark for 2·5 hr. and then an ethyl acetate extract prepared as above.

Saponification. 21-Acetoxy steroids were dissolved in a small volume of 0·4% methanolic $KHCO_3$ (w/v), and the solution was saturated with N_2 and left for 8 hr. at room temperature in the dark (Meyer, 1953). The free steroid was extracted with ethyl acetate and the extract washed with water, filtered through anhydrous Na_2SO_4 and evaporated for chromatography, as above. Little hydrolysis of 3-acetoxy groups occurred with this method.

3-Acetoxy groups were hydrolysed by dissolving the steroid in 0·5–2·0 ml. of 0·067 N-NaOH in 80% methanol and leaving in the dark under N_2 for 21 hr. The steroid was then extracted as above.

Absorption spectra. Absorption spectra in concentrated H_2SO_4 were obtained by the method of Zaffaroni (1950, 1953), care being taken to standardize the time at which each wavelength was read. Both known and unknown steroids were treated with H_2SO_4 after elution from a chromatogram, and compared against a blank tube containing the dried eluate from a similar area of the chromatogram containing no added steroid. Despite these precautions, material giving a strong peak at 320 mμ was troublesome in the eluates from chromatograms run in system 2. Good results were obtained with material from chromatograms run in system 1.

Ordinary ultraviolet spectra were measured in ethanol. All spectra were measured on the Unicam spectrophotometer.

Identification of allotetrahydrocortisol

The schemes of reactions and chromatograms used for this identification are given in Figs. 3 and 4.

Pool 1. Residues of fraction 3 from six 24 hr. urine samples from a patient with mild adrenogenital syndrome were pooled and run on Whatman no. 3 MM paper for 5 hr. in system 1. The unknown reducing substance was eluted and run on Whatman no. 2 paper for 15 hr. in system 1. After elution of the appropriate zone, a fraction of the eluate calculated to contain 25 μg. was oxidized with $NaBiO_3$ and the product run for 16 hr. in system 2. Half the chromatogram was treated with alkaline *m*-dinitrobenzene, which gave a purple spot identical in position and colour with that given by 3α:11β-dihydroxy-5α-androstan-17-one on the same sheet. The appropriate area of the other half of the sheet was eluted and the material acetylated in parallel

with 3α:11β-dihydroxy-5α-androstan-17-one. The esters were run for 14 hr. with system 3. On being treated with alkaline *m*-dinitrobenzene the two esters gave purple spots 32·6 and 32·8 cm. from the origin, as expected for the 3-acetate of the reference steroid.

The remainder of the original substance was acetylated and run in system 2 (single-length run). A reducing substance was detected with blue tetrazolium running just behind the diacetate of tetrahydrocortisol. (Traces of 3α-hydroxy-5β-androstane-11:17-dione were found with the main $NaBiO_3$ oxidation product; these were explained by the presence of traces of tetrahydrocortisone diacetate on the chromatogram of the esters in system 2.)

Pool 2. The unknown substance was obtained as in pool 1 from another patient with mild adrenogenital syndrome and purified by running on Whatman no. 3 MM paper in system 1 for 15 hr. It was then run for 18 hr. on Whatman no. 2 paper in system 1 and treated with blue tetrazolium reagent and with the NaOH fluorescence test. No substance giving any fluorescence with NaOH was found on the chromatogram. On running in system 1 for 36 hr. the substance still gave a single spot with blue tetrazolium reagent.

Pool 3. The unknown substance was prepared as above from the urine of another patient with adrenogenital syndrome. It was then run in parallel with *allo*tetrahydrocortisol for 15 hr. in system 1. After acetylation the substance was run in parallel with *allo*tetrahydrocortisol diacetate in system 2 (single-length run). Both the free compound and its ester moved to positions identical with those of the reference steroids.

A part of the unknown ester was oxidized with chromic acid and run in system 2 (single length) in parallel with *allo*tetrahydrocortisone diacetate (3α:21-diacetoxy-17α-hydroxy-5α-pregnane-11:20-dione). The R_F values of the two substances were identical.

The chromic acid oxidation product of the ester of the unknown substance was then submitted to hydrolysis with methanolic $KHCO_3$ simultaneously with *allo*tetrahydrocortisone diacetate. The products were run in system 2 (single-length run). Both substances gave rise to a reducing substance in the position expected for the 3-monoacetate of *allo*tetrahydrocortisone.

Pool 4. Residues of fraction 3 from five patients with Cushing's syndrome were pooled. The pooled extract was compared with *allo*tetrahydrocortisol by running in system 1 for 36 hr. The urinary reducing substance appeared to be homogeneous and had moved to the same position as the reference steroid.

Pool 5. The unknown substance was isolated from the urine of a patient with adrenogenital syndrome by running fraction 3 on Whatman no. 3 MM paper for 15 hr. with system 1, and eluting. The material was oxidized with sodium bismuthate and part of the oxidation product acetylated in parallel with 3α:11β-dihydroxy-5α-androstan-17-one. The esters were then oxidized with chromic acid and the products were saponified with methanolic NaOH. On running in system 2 (single-length run) both products gave a single spot with alkaline *m*-dinitrobenzene in the position known for 3α-hydroxy-5α-androstane-11:17-dione, and giving the characteristic pinkish colour which partly

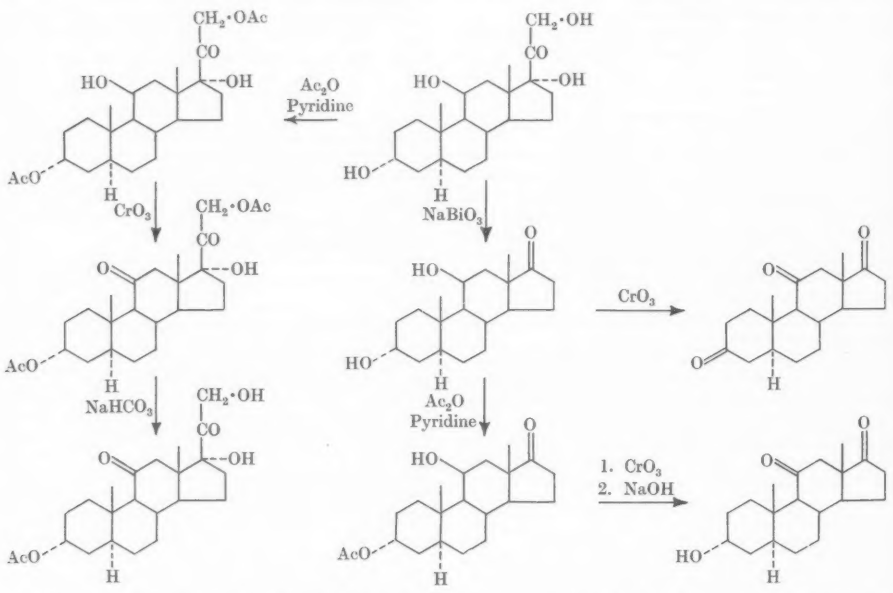

Fig. 3. Scheme of reactions used to identify *allo*tetrahydrocortisol in extracts of urine. In each case the appropriate reference compound was treated with reagents and run on a chromatogram in parallel with the material from urine. In this way relative yields of major and minor products can be compared directly with confidence.

develops in the cold. Traces of androstane-3:11:17-trione and presumed 3α-acetoxy-5α-androstane-11:17-dione were also found with both products.

The remainder of the $NaBiO_3$ oxidation product of the original substance was oxidized with chromic acid for 16 hr. simultaneously with samples of 3α:11β-dihydroxy-5α-androstan-17-one and 3α-hydroxy-5α-androstane-11:17-dione. The three products were then run in system 3 for 15 hr. and the chromatogram was treated with alkaline *m*-dinitrobenzene. All three products gave immediate bluish-purple colours in the cold which intensified on heating, and were at identical distances from the origin. In subsequent tests (Fig. 4) it was shown that the triones isomeric at C-5 are not separated in this solvent system (i.e. 5α-androstane-3:11:17-trione and 5β-androstane-3:11:17-trione).

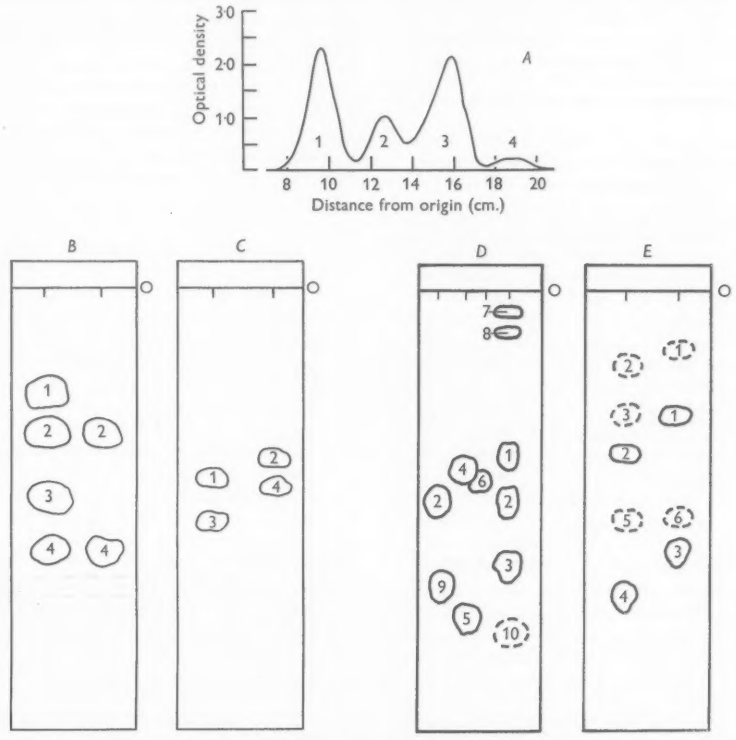

Fig. 4. *A*, Typical scanning record of reducing steroids on a chromatogram of a urinary extract. The strip was equilibrated for 3 hr. and run for 18 hr. in system 1 at room temperature, with Whatman no. 2 paper. 1, tetrahydrocortisol; 2, *allo*tetrahydrocortisol; 3, tetrahydrocortisone; 4, cortisol. Chromatograms *B–E* show typical separations at room temperature. To achieve complete resolution of 5α and 5β epimers, over-run chromatograms (14–18 hr.) were always used. The epimeric (5α- and 5β-)androstanetriones cannot be separated in these systems.

B, 1, tetrahydrocortisol; 2, *allo*tetrahydrocortisol; 3, tetrahydrocortisone; 4, *allo*tetrahydrocortisone (system 1, Whatman no. 2, equilibrated 3 hr., run 18 hr.). O, origin with two tracks of compounds. 2 and 4 run in both tracks.

C, 1, tetrahydrocortisol diacetate. 2, *allo*tetrahydrocortisol diacetate. 3, tetrahydrocortisone diacetate. 4, *allo*-tetrahydrocortisone diacetate (system 2, Whatman no. 2, equilibrated 3 hr., run 4 hr.).

D, 1, dehydro*epi*androsterone. 2, 3α-hydroxy-5β-androstan-17-one. 3, 3α-hydroxy-5α-androstan-17-one. 4, 3β-hydroxy-5α-androstan-17-one. 5, 3β-hydroxy-5β-androstan-17-one. 6, 3α-hydroxy-5β-androst-9(11)-en-17-one. 7, 3α:11β-dihydroxy-5β-androstan-17-one. 8, 3α-hydroxy-5β-androstane-11:17-dione. 9, 6β-hydroxy-3:5-*cyclo*androstan-17-one. 10, 3α-acetoxy-11β-hydroxy-5α-androstan-17-one. Run in system 3 on Whatman no. 2 paper, equilibrated 3 hr.; —, run 16 hr.; - - -, run 14 hr.; O, origin with 4 tracks of compounds.

E, 1, 3α:11β-dihydroxy-5β-androstane-17-one. 2, 3α:11β-dihydroxy-5α-androstan-17-one. 3, 3α-hydroxy-5β-androstane-11:17-dione. 4, 3α-hydroxy-5α-androstane-11:17-dione. 5, 5α-androstane-3:11:17-trione. 6, 5β-androstane-3:11:17-trione. O, origin with two tracks of compounds. Conditions were as for *D*; but —, run 16 hr.; - - -, run 4 hr.

Absorption spectra. Samples of the unknown substance from pools 1–3 were run on specially washed paper (see Materials) and eluted. The diacetate and the chromic acid oxidation product of the diacetate were treated similarly, in parallel with the appropriate reference steroids. Details of the spectra in H_2SO_4 are given in Table 1. Not unexpectedly the agreement between the spectra of the diacetates and their reference steroids was unsatisfactory because of the impurities, described above, in eluates from chromatograms run in system 2, and also because the resolution obtained with the single-length run is not great. The agreement between the free compounds, in which a long run in system 1 could be made, was, however, good. As expected from the absence of fluorescence with NaOH, the original substance in ethanol gave no absorption peak in the ultraviolet.

Table 1. *Absorption spectra in concentrated sulphuric acid*

The diacetates were isolated from chromatograms run with system 2, which gave unexpectedly high blank values with sulphuric acid in the range 240–360 mμ; the differences between reference steroid and isolated urinary material were therefore not considered significant.

Compound	$\lambda_{max.}$ (mμ)
Tetrahydrocortisol	245, 330, 410, 505
*allo*Tetrahydrocortisol (I)	240, 330, 410, 505
Urinary material	240, 330, 410, 505
Diacetate of (I)	240, 285, 342, 415
Diacetate of urinary material	235, 320, 410
*allo*Tetrahydrocortisone diacetate	275, 320, 410
CrO_3 oxidation product of the diacetate of urinary material	240, 317, 410

RESULTS

Excretion rate of allo*tetrahydrocortisol*

Table 2 summarizes the excretion rates of the three reducing steroids believed to be derived from cortisol in man. It is seen that *allo*tetrahydrocortisol is excreted in quantities that are far from insignificant, although less than those of the other two steroids. It is also clear that the ratio between the (5β)-pregnane metabolites and *allo*tetrahydrocortisol [(5α)-pregnane] is not greatly different in the different groups of subjects studied here.

In Fig. 5 the results are plotted as a regression of the excretion rates of the (5α)-pregnane metabolite on the sums of the excretion rates of the (5β)-pregnane metabolites in each of 52 urine samples. If y is the excretion rate of *allo*tetrahydrocortisol and x the sum of the excretion rates of tetrahydrocortisol and tetrahydrocortisone, the regression of y on x is given by the equation $y = 0{\cdot}199x - 0{\cdot}1625$. The regression coefficient is 0·924 ($P \leqslant 0{\cdot}001$); the deviation of the point of intersection of the x and y axes ($a = -0{\cdot}1625$) is not significant ($P \geqslant 0{\cdot}5$).

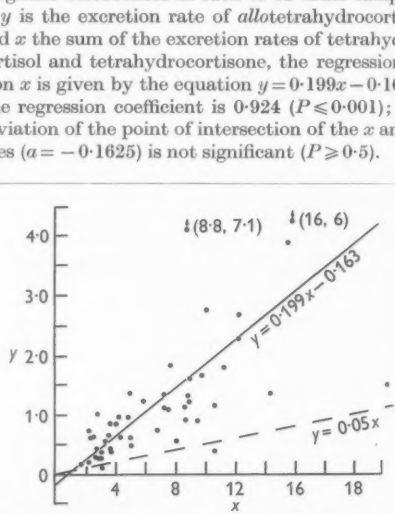

Fig. 5. Regression of excretion rates of *allo*tetrahydrocortisol (y) on the summed excretion rates of tetrahydrocortisol and tetrahydrocortisone (x), in mg./24 hr. Solid line is for $y = 0{\cdot}199x - 0{\cdot}163$. Interrupted line is for $y = 0{\cdot}05x$, as expected from Dorfman's (1954*a*) theory.

Table 2. *Excretion rates of the main* 3:4:5*-tetrahydro metabolites of cortisol*

Rates (mg./24 hr.) are given as means with ranges in parentheses and are corrected for the analytical losses usual in the method of estimation. The mean ratios 5α(H) metabolite/5β(H) metabolites are given in the last column.

Subjects	No.	Tetrahydrocortisol mean (range)	*allo*Tetrahydrocortisol mean (range)	Tetrahydrocortisone mean (range)	$\frac{5\alpha(H)}{5\beta(H)}$
Normal men and women	14	2·0 (1·1–4·8)	0·8 (0–2·8)	3·4 (1·0–10·4)	0·165
Idiopathic hirsutism (women)	7	1·8 (0·9–4·5)	0·7 (0·3–1·6)	2·3 (1·1–4·6)	0·202
Cushing's syndrome (mild)	4	4·15 (3·5–5·3)	1·2 (0·9–1·7)	4·85 (3·9–5·7)	0·42
Adrenogenital syndrome	11	2·0 (0·4–3·3)	0·75 (0·2–1·8)	2·7 (1·1–4·5)	0·173
Obesity and hypertension	1	1·7	0·6	1·9	0·166
Various women with hirsutism or adrenogenital syndrome on oral cortisone	10	3·55 (1·8–4·0)	1·2 (0·6–2·3)	6·0 (1·8–10·5)	0·162
Women with hirsutism or adrenogenital syndrome on ACTH gel, intramuscular	5	10·6 (4·7–18·0)	5·7 (1·3–12·4)	9·9 (4·3–17·7)	0·25

While the regression equation and coefficient indicate a highly significant correlation of the excretion of *allo*tetrahydrocortisol with that of tetrahydrocortisol and tetrahydrocortisone, it should be noticed that this is heavily weighted towards significance by a few figures from subjects excreting large quantities of these steroids. Furthermore, separation of the subjects into groups according to their clinical state and treatment reveals significant differences ($P \leqslant 0{\cdot}025$) between the ratio 5α-(H) compound:5β-(H) compounds of the different groups, if these are tested by a test for ordered alternatives rather than against a null hypothesis (Jonckheere, 1954). Thus this ratio is lower in a small group of cases of Cushing's syndrome, and higher among the hirsute females, than in the normal group (mean ratios 0·142, 0·202, 0·165). A striking finding was that treatment of the hirsute group with cortisone lowered this ratio to normal (mean 0·162), whereas treatment with ACTH increased it still further (mean 0·250), both differences significant at $P \leqslant 0{\cdot}02$. There is no question of a systematic error underlying this finding, since the total excretion rates were large in both groups, the mean total excretion rates (reducing steroids) of both groups being larger than in the untreated patients. There are two possible explanations of this finding, but further work is needed to decide between them with confidence.

The first is that the phenomenon is due to a difference in the metabolism of cortisol (or cortisone) by the three groups of subjects, which is shown up by oral administration of cortisone. The major part of the steroid is absorbed via the portal system when administered orally, and if the enzyme system producing the 5α-(H) metabolite was of relatively limited activity, in contrast to that producing the 5β-(H) metabolite, the proportion of 5α-(H) metabolite would fall during oral administration of its precursor. An alternative hypothesis of this type, which would be in accord with the very high substrate specificity of Δ^4-3-oxo steroid reductases (Tomkins, 1957), would be that cortisol, but not cortisone, was reduced to the 5α-(H) metabolite. This type of hypothesis would not, without elaboration, explain the increased proportion of *allo*tetrahydrocortisol excreted during treatment with ACTH. A second type of explanation, accounting for this and the other observations, would be that *allo*tetrahydrocortisol was secreted by the adrenal cortex, in addition to being a metabolite of cortisol, and that the proportion secreted was larger than normal in the hirsute women and lower than normal in the cases of Cushing's syndrome. This hypothesis seems to us rather doubtful but receives some support from the fact that *allo*tetrahydrocortisol and its 11-oxo derivative are found in extracts of adrenal glands, and are the only 3α-OH-5α(H) pregnane derivatives in such extracts (Reichstein, 1936).

None of these three reducing steroids were found in the urine of two patients with Addison's disease, or in the urine of a man who had been bilaterally adrenalectomized, when they had been treated with deoxycorticosterone and salt. In all three cases all three steroids appeared in the urine when cortisone (25–50 mg./day) was given.

DISCUSSION

Identification of allo*tetrahydrocortisol*

The scheme employed has been summarized in Fig. 3, and the chromatograms of the substances and their derivatives involved in this study are shown in Fig. 4. Following the general lines suggested before (Bush & Sandberg, 1953; Bush, 1954) seven derivatives were prepared and compared chromatographically with reference steroids. Not only were the mobilities of the substance and all seven derivatives identical in the three solvent systems employed, but several characteristic details of these mobilities and the reactions of the derivatives are worth emphasizing. Thus proportions of minor products of oxidation, esterification and saponification were exactly the same for reference steroid and urinary material in each case. Highly characteristic also was the fact that, although the free 3α-OH-5β(H) steroids were slightly less polar than the related 3α-OH-5β(H) isomers, the opposite was true for their diacetates. Other features were the reactions given by the 17-oxo steroid derivatives with alkaline *m*-dinitrobenzene. 3α:11β-Dihydroxy-5α-androstan-17-one gave a rather weak purple colour only after heating; 3α-hydroxy-5α-androstane-11:17-dione [like its (5β)-*cis–trans* isomer] gave a strong pinkish colour developing partly in the cold; (5α)-androstane-3:11:17-trione gave a strong bluish colour developing partly in the cold. In all these details the derivatives of the urinary substance agreed exactly with the known reference steroids.

The series of reactions used here subjected each substituent in the steroid nucleus in turn to one or more unequivocal reactions. In this way changes in colour reaction and chromatographic mobility could be interpreted with considerable confidence. The sequence of reactions given in Fig. 3 is based on the reasonable assumption that the material is a steroid, and the reasonable induction from previous work that within limits the identification of steroids by chromatographic mobility and colour reactions is sound (Zaffaroni, 1953; Savard, 1954; Bush, 1954). It is, however, possible to consider the evidence largely without relying upon the assumption that the material is a steroid (see Table 3).

Previous workers have failed to detect *allo*tetrahydrocortisol in human urine, although it has been seen that its excretion rate, though small, is not insignificant compared with tetrahydrocortisol and tetrahydrocortisone, which are well known as urinary metabolites (Schneider, 1950, 1952; Baggett, Kinsella & Doisy, 1953; de Courcy *et al.* 1953; Lieberman *et al.* 1953; Romanoff, Wolf, Constandse & Pincus, 1953; Romanoff & Wolf, 1954; Cope & Hurlock, 1954; Richardson *et al.* 1955). [After the present paper had been submitted for publication Romanoff, Seelye, Rodriguez & Pincus (1957) announced the identification of *allo*tetrahydrocortisol in the urine of normal humans and

Table 3. *Summary of steps in the identification of urinary* allo*tetrahydrocortisol*

In Fig. 3 the series of reactions used is given in terms of the actual steroid derivatives expected from such reactions, and identification of each derivative is based on identical chromatographic mobilities and colour reactions. In this table each functional group is considered separately and without reference to the identity of the derivatives themselves.

Side chain

(1) Oxidized by $NaBiO_3$ to a carbonyl compound.
(2) Oxidized by alkaline blue tetrazolium rapidly in the cold.
(3) Not oxidized to a carbonyl compound by periodate.
(4) Not oxidized by chromic acid after acetylation.
(5) Oxidized by alkaline blue tetrazolium rapidly in the cold after acetylation.
(6) No Zimmermann reaction.
(7) $NaBiO_3$ oxidation product gives purple colour with Zimmermann reagent characteristic of 17-oxo steroids.

The reducing side-chain is linked to the nucleus with a tertiary hydroxyl group (1, 7) but does not contain a hydroxyl group adjacent to this (3). The reducing part contains an easily esterifiable group (4), the ester being labile to cold alkali (5). The only known group meeting all requirements is a dihydroxyacetone side-chain.

11β-Hydroxyl group

(8) Oxidation with CrO_3, after acetylation, causes an increase in R_F of the expected amount with the original substance, and with the derivative given by $NaBiO_3$ oxidation.
(9) The oxidizable group is not protected by acetylation in the cold.
(10) The $NaBiO_3$ oxidation product gives a weak purple colour with the Zimmermann reagent. Oxidation of the group in question gives a derivative giving a strong pink colour in the cold with the Zimmermann reagent.

A strongly hindered secondary hydroxyl group is responsible for these properties (8, 9). All these (8, 9, 10) are highly characteristic of the 11β-hydroxyl group in the natural steroids. No other hydroxyl group in the steroid nucleus has all these properties.

3-Hydroxyl group

(11) An easily esterifiable group exists on the nucleus, since acetylation of the substance causes an increase in R_F greater than that accounted for by the group in the side chain.
(12) The increase in R_F of the substance, and of its $NaBiO_3$ oxidation product, after acetylation, are too small to be due to more than one such group.
(13) The esterified group is hydrolysed by NaOH in the cold but not by $KHCO_3$.
(14) The group is oxidized by CrO_3 to a less polar group which causes a considerable blueing of the colour given by the Zimmermann reagent.

One acetylatable secondary hydroxyl group exists on the nucleus (11, 12, 14). In the steroids only position 3 will satisfy (14). Position 3 satisfies (13).

3α-OH-(5α)H configuration

(15) Oxidation of the second nuclear secondary hydroxyl group causes an increase in R_F which is too small for 3β-OH-5α(H) or 3α-OH-5β(H) and too large for 3β-OH-5β(H) configurations. This increase in R_F is exactly that found with the 3α-OH-5α(H) configuration.
(16) The increase in R_F on acetylation is too small for the 3α-OH-5β(H) configuration.

Only the 3α-OH-5α(H) configuration satisfies all requirements (14, 15, 16).

Absence of other substituents

(17) The substance and all its recognizable derivatives are not extracted by alkali (Na_2CO_3) or acid (HCl) from ethyl acetate.
(18) No derivative given by CrO_3 oxidation has a lower R_F than the starting material of such an oxidation.
(19) The substance has no absorption maximum in visual or ultraviolet range.
(20) R_F values of the substance and all derivatives satisfy the existence of a steroid nucleus and the polar groups shown by the reactions above.

Carboxyl (17), basic (17), phenolic (19), other tertiary hydroxyl groups (18), and conjugated systems (19) are excluded. Exceptionally stable ester groups or very weakly basic groups are not excluded by the reactions above; it is highly unlikely that such groups would not be revealed by differences in R_F or colour reactions of one or more of the seven derivatives prepared.

schizophrenics. Their findings are very similar to those reported here.] Although this could be due to a variety of differences in technique, it seems most likely that incomplete separation from tetrahydrocortisol on chromatograms, coupled with the identical spectra in sulphuric acid of these two *cis–trans* isomers, was responsible. Richardson *et al.* (1955) have pointed out already that the latter factor might have vitiated their identification of tetrahydrocortisol; inspection of their scanning curves suggests that the two epimers would not have been separated. Similarly, the single-length chromatograms of de Courcy *et al.* (1953) were not sufficient to separate either the free compounds or their acetates when mixed in the proportions usual in urine extracts. However, the presence of the (5α)-epimer would have been demonstrated, even if it had not been separated from tetrahydrocortisol, if oxidation with sodium bismuthate had been carried out, since the analogous *cis–trans* epimers among the C_{19} steroids are more easily separated.

Metabolic origin of allo*tetrahydrocortisol*

The direct evidence from patients with adrenal insufficiency, and the significant correlation of the excretion rate of this substance with those of tetrahydrocortisol and tetrahydrocortisone (which are known metabolites of cortisone and cortisol), under a wide variety of conditions, suggest strongly that *allo*tetrahydrocortisol is itself a metabolite of cortisol. The mean ratio of *allo*tetrahydrocortisol (tetrahydrocortisol + tetrahydrocortisone) in the fifty-two urine samples was 0·16 (S.D. 0·08). While this is about three times that expected from the theory of Dorfman (1954*a*), it does not contradict the general rules he has described. However, the wide scatter of the values of this ratio, when measured in single 24 hr. specimens of urine, is similar to that found with other classes of steroid with the same *cis–trans* isomerism (Gallagher, 1954*a*, *b*; Fukushima, Dobriner & Gallagher, 1954; Dorfman, 1954*b*, 1955).

It will be seen from Fig. 4 that *allo*tetrahydrocortisone would not be separated from cortisol on our routine quantitative chromatograms. However, we have evidence that the former substance was not present in more than traces in the urine samples studied here. Measurement of the excretion rate of cortisol was made on many of the specimens of this series, by separate chromatograms and the NaOH fluorescence test (Bush, 1953), by means of visual matching with standards (error ± 20 %). In every case where reducing material was found in this zone, on chromatograms examined by the quantitative blue tetrazolium method, it could all be accounted for by the amount of cortisol determined by the fluorescence method. In most cases the amount of reducing material in this zone was just at or below the limit of measurement with blue tetrazolium (i.e. with 0·01 of 24 hr. sample), so that even in samples where cortisol was not measured separately the amount of *allo*tetrahydrocortisone must have been extremely small. It seems unlikely that the ratio of the 11-oxo to the 11-hydroxyl compound can have exceeded 0·05 in any of our subjects.

This negligible quantity of the 11-oxo compound is remarkable (Bush, 1956) in that the same phenomenon occurs with two other types of 5α(H)-3α:11β-dihydroxy steroids, in contrast with their 5β(H) *cis–trans* isomers. Thus 3α:11β-dihydroxy-5α-androstan-17-one and 3α:11β:21-trihydroxy-5α-pregnan-20-one are found in human urine, but their 11-oxo forms are not present in easily demonstrable quantity (Gallagher, 1954*b*; Dorfman, 1954*b*; Kappas & Gallagher, 1955; Kappas, Dobriner & Gallagher, 1955; Engel, Carter & Springer, 1954; Richardson *et al.* 1955). On the other hand, with the related (5β)-H *cis* isomers the 11-oxo form predominates in a ratio of 2–4. If the enzyme reducing 11-oxo steroids to 11β-hydroxy steroids found in rat-liver homogenates by Hubener, Fukushima & Gallagher (1956), is present in man, then these findings would be explained if the enzyme were capable of acting on 3α-OH-5α(H) steroids as well as on Δ^4-3-oxo steroids. This has not yet been tested, but Hubener *et al.* (1956) have shown that this enzyme cannot attack 3α-OH-5β(H) steroids, which would afford a possible explanation of the predominance of the 11-oxo forms of the 3α-OH-5β(H) steroid metabolites. [Since this paper was submitted, it has been shown that 3α-hydroxy-5α-androstane-11:17-dione is partially reduced to the 11-alcohol in man (Bush & Mahesh, 1957).] The only other explanation for this phenomenon would be that the enzyme responsible for reduction of Δ^4-3-oxo steroids to 3α-OH-5α(H) steroids did not act *in vivo* on 11-ketones, although it acted on 11-deoxy steroids and on 11β-alcohols; while possible, this seems very much less likely than the first hypothesis.

SUMMARY

1. A reducing substance was noticed on chromatograms of urine extracts which had an R_F just greater than that of 3α:11β:17α:21-tetrahydroxy-5β-pregnan-20-one. It was almost invariably present in urine from normal subjects, and from patients with various forms of hyperadrenalism, but absent from patients with Addison's disease or those who had undergone adrenalectomy, unless the latter patients were treated with cortisone.

2. Derivatives were prepared of the reducing substance after isolation from chromatograms, in parallel with known steroids, and identified by

means of chromatographic mobility, colour reactions, and spectra in sulphuric acid. In all respects the substance was found to be identical with 3α:11β:17α:21-tetrahydroxy-5α-pregnan-20-one.

3. Evidence was obtained that the reducing substance is a normal metabolite of hydrocortisone and cortisone in man, previously unrecognized because of its similarity to its 5β-epimer in many respects. This modifies some of the earlier conclusions of Dorfman (1954*a*).

4. Further work is needed to elucidate some unusual features of the excretion rate of the reducing substance in women suffering from various forms of adrenal virilism and hirsutism.

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Observations on the Respiratory Metabolism of Rat-Adrenal Cortex

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Study of the respiratory and carbohydrate metabolism of adrenal-cortex tissue may be expected to yield information of interest in connexion with the growth and secretory function of the gland. Indications of the quantitative changes in oxidative activities which may take place under the influence of regulatory hormones have already been given. Carpenter, Macleod & Reiss (1946) showed that the oxygen uptake by rat-adrenal cortices *in vitro* in Ringer–glucose solution was higher with animals treated with adrenocorticotrophic hormone than with normal animals. Sourkes & Heneage (1951) found that homogenates of whole adrenals from cortisone-treated rats showed decreased rates of endogenous respiration, and decreased ability to oxidize various intermediates of the citric acid cycle.

This investigation was begun in order to obtain information on the respiration of rat-adrenal-cortex tissue in the presence of various substrates. Some observations on the glycolytic activities of the tissue were also made.

METHODS

Preparation of tissue. Female rats of about 200–250 g. body wt. were used exclusively. They were killed by decapitation, the carcasses were exsanguinated and the adrenals were rapidly removed, cleaned from fat, opened laterally with fine scissors and carefully bisected. The medullae could then be dissected out, and though a part of the zona reticularis is unavoidably lost, these demedullated bisected adrenals may be regarded as intact adrenal-cortex tissue. According to Carpenter *et al.* (1946) cortices prepared in this way satisfy the conditions required for diffusion in manometric experiments. The tissue was washed once by placing it in ice-cold Ringer solution, then blotted and transferred to the Warburg vessels.

Medium. The modified Ringer solution of Krebs & Henseleit (1932) was used, containing as buffer either 0·05 M-phosphate (pH 7·4) or 0·027 M-bicarbonate.

Measurements of oxygen uptake. These were carried out by the Warburg manometric technique at 37°. The tissue was allowed to respire in Krebs–Ringer–phosphate solution with the appropriate substrate in an atmosphere of O_2, CO_2 being absorbed by KOH soln. on filter paper in the central compartment of the vessel. In some preliminary experiments the bisected cortices were mixed indiscriminately, but in all the later experiments it was found advantageous to distribute the halves from a number of adrenals between a pair of Warburg vessels in such a way that the half of an adrenal in one vessel was matched by the corresponding other half in the second vessel. Portions of tissue distributed between a pair of vessels in this way are referred to as 'paired bisects'. Six bisects per vessel were normally used, and measurements of uptake of O_2 were carried out for 3 hr. The tissue in each vessel was then removed, blotted with filter paper and dried at 100°.

Glycolysis. This was measured at 37° in Krebs–Ringer–bicarbonate solution in an atmosphere of $CO_2 + N_2$ (5:95) or $CO_2 + O_2$ (5:95). In the anaerobic experiments yellow phosphorus was placed in the central compartment of the vessel. Measurements were made at 37° for 2 hr. The pieces of tissue were then removed for determination of dry wt., and the remaining liquid was centrifuged. Lactic acid was estimated on 1 or 2 ml. samples by the method of Barker & Summerson (1941).

Respiratory quotient. This was determined at 37° both by Warburg's (1914) direct method (correction being made for retention of CO_2) and by the method of Dickens & Šimer (1930*a*), with incubation periods of 2 and 3 hr. for each method.

Materials. D-Glucosamine hydrochloride and hexose diphosphate were obtained commercially. The dicalcium salt of the latter was converted into the sodium salt before use.

RESULTS

Endogenous respiration

In the first experiments, each vessel contained six bisects (total dry wt. 20–30 mg.) which were mixed indiscriminately. Later, the use of correctly paired bisects was tried and was found to give more consistent duplicates. This method was therefore used in all the subsequent work. Representative results of a series of experiments showing the variations in endogenous respiration between sets of paired bisects, with different numbers of bisects, are given in Table 1.

The values for Q_{O_2} measured with different amounts of tissue are reasonably close, and this perhaps offers some support for the view of Carpenter *et al.* (1946) that the demedullated bisects of rat adrenals are suitable as a form of tissue with which to study the respiratory metabolism of surviving adrenal cortex.

Effects of various substances on the respiration

Each substrate was added to a set of paired bisects, with the corresponding set of paired bisects acting as the control. The results are shown in Table 2.

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Table 1. *Endogenous respiration of paired rat-adrenal cortices in Krebs–Ringer–phosphate medium* (pH 7·4) *in oxygen at* 37°

No. of bisects/vessel	Total dry wt. of tissue (mg.)		$-Q_{O_2}$ 1st hr.		2nd hr.		3rd hr.	
	1st halves	2nd halves	1st halves	2nd halves	1st halves	2nd halves	1st halves	2nd halves
8	30·9	24·6	3·1	3·4	2·1	2·3	1·03	1·3
8	22·9	27·6	3·2	3·3	1·6	1·8	0·8	0·9
6	17·6	18·3	3·5	3·4	1·5	1·7	0·6	0·7
4	14·4	11·2	3·8	3·5	2·0	2·3	0·9	0·9
2	11·3	10·2	2·2	2·2	0·9	0·6	0·3	0·5

Table 2. *Influences of various substrates on the respiration of paired rat-adrenal cortices in Krebs–Ringer–phosphate medium* (pH 7·4) *in oxygen at* 37°

Substrate and concn. (M)	$-Q_{O_2}$ 1st hr.		2nd hr.		3rd hr.	
	Without substrate	With substrate	Without substrate	With substrate	Without substrate	With substrate
Glucose, 0·01	2·9	4·1	1·3	4·0	0·6	3·7
Fructose, 0·01	2·5	3·0	1·3	1·7	0·5	1·0
Galactose, 0·01	2·4	3·1	1·6	2·4	0·7	1·5
Lactose, 0·01	2·8	3·2	1·6	2·0	0·6	1·0
Glycogen, 0·1%	3·2	2·8	1·5	2·4	1·1	1·5
D-Glucosamine, 0·02	2·7	5·0	1·3	4·1	0·7	3·0
Sodium lactate, 0·02	2·7	5·1	2·4	4·9	1·1	4·1
Sodium succinate, 0·02	4·5	12·1	3·4	7·4	2·6	7·8
Sodium pyruvate, 0·02	3·8	4·8	2·2	3·5	1·0	2·6
Sodium α-oxoglutarate, 0·02	3·4	4·9	1·8	2·8	0·7	1·8
Sodium α-glycerophosphate, 0·01	2·5	3·4	1·7	1·7	0·9	0·9
Sodium glutamate, 0·01	3·5	3·9	1·8	2·5	1·0	1·6

Of the carbohydrates tried, glucose was by far the most effective in enhancing respiration. The respiration without substrate showed the usual steady decline over the 3 hr. period, but in the presence of glucose it remained practically constant during that time, the effect being particularly marked during the third hour. Fructose, galactose and lactose produced much lower effects. Glycogen produced a positive effect in the second hour only, and this is of doubtful significance. The respiration rate in the presence of glucose is comparable with the rates quoted by Carpenter *et al.* (1946), i.e. $-Q_{O_2}$ 2·82–4·18 for normal rats. There was a considerably increased uptake of O_2 in the presence of D-glucosamine. Of the non-carbohydrates, succinate produced the greatest effect; pyruvate, α-oxoglutarate and lactate also increased the uptake of O_2 markedly. α-Glycerophosphate produced a positive effect in the first hour, and glutamate caused a definite, though small, increase, in agreement with the observation of Sourkes & Heneage (1951).

The following substances, at the molar concentrations indicated, increased the rates of tissue respiration by less than 10% over the first and second hours, and by less than 20% in the third hour: sucrose (0·01), hexose diphosphate (0·02), the sodium salts of malic, citric, acetic, butyric, oleic and gluconic acids (0·02), alanine (0·02), choline (0·03), glycerol (0·01), ethanol (0·02) and cholesterol (saturated). The effects of these substances are accordingly regarded as insignificant. The results for citrate, malate, acetate and butyrate are at variance with those of Sourkes & Heneage (1952), who found that all of these substances were oxidized by ox-adrenal slices, and that citrate and malate were oxidized by homogenates of rat and ox adrenals.

Aerobic and anaerobic glycolysis

In view of the high rate of oxidative catabolism of glucose, it was of interest also to investigate aerobic and anaerobic lactic acid formation by the paired-bisect technique. Results are shown in Table 3. (Figures for Q_{CO_2} for the aerobic experiments are not included because the differences in manometer-level readings were so small that the calculations for CO_2 output cannot be regarded as accurate.) Close agreement was obtained between sets of paired bisects for endogenous-acid formation both in terms of CO_2 output and of lactic acid. In the presence of glucose or hexose diphosphate, the anaerobic output of CO_2 was markedly increased, and the figures thus obtained for $Q^{N_2}_{CO_2}$ are similar to those reported by Dickens & Šimer (1930*b*) for liver and

Table 3. *Aerobic and anaerobic acid production by paired rat-adrenal cortices in Krebs–Ringer–bicarbonate medium*

Tissues were respiring in either CO_2+O_2 or CO_2+N_2 at 37°.

Conditions	Substrate	$Q^{N_2}_{CO_2}$ 1st hr. Without substrate	$Q^{N_2}_{CO_2}$ 1st hr. With substrate	$Q^{N_2}_{CO_2}$ 2nd hr. Without substrate	$Q^{N_2}_{CO_2}$ 2nd hr. With substrate	Lactic acid (μg./mg. dry wt./2 hr.) Without substrate	Lactic acid (μg./mg. dry wt./2 hr.) With substrate
Anaerobic	Glucose, 0·01 M	2·1	3·7	0·9	2·3	4·1	14·2
Anaerobic	Hexose diphosphate, 0·01 M	1·2	3·6	1·2	4·6	2·6	13·6
Anaerobic	Glycogen, 0·2%	2·1	1·3	1·1	1·2	1·7	2·4
Aerobic	Glucose, 0·01 M	—	—	—	—	1·9	6·2

Table 4. *Respiratory quotients of paired rat-adrenal cortices at 37°*

Method	Incubation period (hr.)	Respiratory quotients: Without glucose	Respiratory quotients: With glucose (0·01 M)
Warburg direct	2	0·77	0·74
Warburg direct	3	0·73	0·76
Dickens & Šimer (1930*a*)	2	0·68	0·84
Dickens & Šimer (1930*a*)	3	0·70	0·71

Table 5. *Comparison between endogenous respiration rates of intact adrenal cortices and homogenized adrenal cortices*

Tissues were respiring in Krebs–Ringer–phosphate medium, pH 7·4, in O_2 at 37°.

	$-Q_{O_2}$ (μl. of O_2/mg. wet wt.): Intact cortices	$-Q_{O_2}$ (μl. of O_2/mg. wet wt.): Homogenized cortices
1st hr.	0·44	0·06
2nd hr.	0·40	0·03
3rd hr.	0·29	0·02

kidney. Hexose diphosphate seemed to be slightly though not significantly more active than glucose in stimulating CO_2 output, but the figures for estimated lactic acid were not appreciably different. With glycogen as substrate, neither the CO_2 nor lactic acid content was significantly increased. Lactic acid formation under aerobic conditions in the presence of glucose was comparable with that reported by Carpenter *et al.* (1946), and it appears to be somewhat high in relation to the results obtained under anaerobic conditions.

Respiratory quotient

The relatively high rates of endogenous respiration and the low rates of anerobic glycolysis in the presence of glucose made it of interest to obtain further information regarding the importance of glucose as an oxidizable substrate, and measurements of the respiratory quotients, in the presence and absence of glucose, were undertaken. The results are shown in Table 4. The quotients, both with and without glucose, were well below unity in every case, and in most cases had values which may be regarded as consistent with a fat metabolism.

Comparison between endogenous respiration of bisects and homogenates

The rates of endogenous respiration for the first hour were distinctly higher than those previously reported by Sourkes & Heneage (1952). These workers found Q_{O_2} values of $-1{\cdot}1$ (on a calculated dry wt. basis) for rat-adrenal homogenates and of $-2{\cdot}0$ for ox-adrenal-cortex slices. It seemed probable that the respiratory activity of the rat tissue, without addition of substrates, was largely lost on homogenizing. A direct comparison was therefore made between the endogenous respiration of bisects and homogenates. One set of paired bisects was used intact, after weighing on a torsion balance. The other set was also weighed wet, then homogenized in the minimum volume of Krebs–Ringer–phosphate medium and finally transferred, with washing, to a Warburg vessel, and made up to the appropriate volume. Each vessel was gassed with O_2, and the respiration measured as usual. Typical results are shown in Table 5. It is evident that homogenization much decreases the ability of the tissue to oxidize its endogenous substrates, the rate of endogenous respiration being reduced to less than 10% of the value for intact tissue.

Depletion of endogenous substrates

The presence of appreciable amounts of endogenous oxidizable material may mask the effects produced by added substrates; it is possible that the differences between the present results and those of Sourkes & Heneage (1952) might be due to this. It was decided to determine whether fresh, intact tissue could be depleted of its endogenous substrates by pretreatment with O_2 at 37°. This technique was

found to be of value in the study of the oxidative metabolism of the mammary gland (Terner, 1952). One set of paired bisects was treated with O_2 for 1 hr. at 37° as described by Terner, while the other set was kept in ice-cold Krebs–Ringer solution. The two sets of bisects were then transferred to Warburg vessels containing the buffered medium, the vessels were gassed with O_2, and the respiration rates measured. The pretreatment with O_2 resulted in a substantial fall in endogenous respiration to about 50 % in the first hour and 37 % in the second hour. In a second experiment, $-Q_{O_2}$ values for pretreated tissue were 1·55 (first hour) and 0·7 (second hour), and these increased to 2·4 (first hour) and 2·15 (second hour) in the presence of 0·01 M-glucose, indicating that the depleted tissue is still able to oxidize glucose. It is probable that the depletion technique could be used with advantage in studying the oxidative metabolism of this tissue.

DISCUSSION

Of the carbohydrates studied, glucose is evidently the most readily utilized. Though there is some evidence that fructose, galactose and lactose may be oxidized, their rates of oxidation are in any case considerably lower than that for glucose. Apart from oxidative catabolism, glucose appears to be utilizable by an anaerobic glycolytic mechanism, presumably through a series of reactions on the lines of the Embden–Meyerhof scheme, since lactic acid is formed from hexose diphosphate to approximately the same extent as from glucose. There is little or no glycolytic activity with glycogen as substrate; the relative inefficiency of glycogen, compared with glucose, in promoting glycolysis in adrenal-cortex tissue has already been observed by Haarmann (1932). The glycolytic rates in the presence of glucose, however, are not high, and it seems likely that the anaerobic catabolism of glucose is of minor importance. It is of interest in this connexion that Glock & McLean (1954) found that glucose 6-phosphate dehydrogenase and 6-phosphogluconate dehydrogenase activities are very high in adrenal-cortex tissue. Kelly, Nielson, Johnson & Vestling (1955) have deduced from this, and from other evidence, that the direct oxidative pathway of glucose catabolism is of particular importance in the adrenal cortex.

These observations have a bearing on the energy requirements of the gland in connexion with steroid synthesis. As far as is known, the adrenal cortex does not store glycogen and would depend, in large measure, on the rapid oxidation of circulating glucose in the blood. The high rates of oxidation of some of the intermediates of carbohydrate metabolism found in the present investigation, and found by Sourkes & Heneage (1951, 1952), are in accordance with this. A possible connexion between carbohydrate metabolism and corticosteroid synthesis has previously been indicated by Vogt's (1951) finding that adenosine triphosphate and creatine phosphate caused a marked increase in the secretion of corticosteroids by perfused adrenals of dogs.

At the same time, the low values found for the respiratory quotients, both in the presence and absence of glucose, would seem to point to the oxidation of much fat, although the present investigation did not produce evidence that added fatty acids are oxidized by the tissue under the particular conditions used.

The observation that glucosamine is oxidized by the adrenal cortex is of interest, since this substance is known to be oxidized at appreciable rates by only three other tissues: brain cortex, kidney cortex and testis (Lutwak-Mann, 1941). Comparing the present measurements with those reported by Lutwak-Mann, the rate of oxidation of glucosamine for adrenal-cortex tissue appears to be comparable with that for brain cortex, but lower than that for kidney cortex and testis.

SUMMARY

1. The influence of various substances on the respiration of intact rat-adrenal cortex has been studied.

2. Glycolysis in this tissue is stimulated by glucose and hexose diphosphate under anaerobic conditions, and by glucose under aerobic conditions.

3. The respiratory quotient, in the presence and absence of glucose, is consistent with the oxidation of much fat.

4. The endogenous respiration is considerably reduced when the tissue is homogenized, or when it is pretreated with oxygen at 37°.

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[*The Editors of the Biochemical Journal accept no responsibility for the Reports of the Proceedings of the Society. Abstracts of papers read are published as received from the authors.*]

PROCEEDINGS OF THE BIOCHEMICAL SOCIETY

The **364th Meeting** *of the Biochemical Society was held in the Washington Singer Laboratories of the University of Exeter on Friday, 19 July 1957, starting at* **11.15** *a.m., when the following papers were read:*

COMMUNICATIONS

Some Properties of the Larval Gut Proteinase of the Blowfly, *Calliphora erythrocephala*. By W. A. L. Evans (introduced by J. Pryde). (*Department of Zoology and Comparative Anatomy, University College, Cardiff*)

The proteinase was obtained by aqueous extraction of dried alimentary tracts of mature larvae. After the addition of toluene the extract was dialysed against running tap water for 24 hr. Dialysed extracts showed approx. 25% greater proteinase activity than non-dialysed extracts. The reaction mixtures comprised buffer (either phosphate or borate), suphanilamide-azocasein as substrate and enzyme extract. Proteinase activity was estimated colorimetrically by the method of Charney & Tomarelli (1947).

The pH-activity curve was similar to that of other blowfly larvae (Hobson, 1931), and the pH optimum at 7·8 corresponded closely to the pH of the anterior midgut and posterior midgut. Using gelatin as substrate and the copper method of Pope & Stevens (1939) to measure hydrolysis, no proteinase activity was detected at pH values below 3·75.

The velocity-substrate concentration relationship was typical. By suitably transposing the Michaelis–Menten equation several graphical methods are available for evaluating K_m (Lineweaver & Burk, 1934; Dixon, 1953; Hofstee, 1953; Eadie, 1953). None of the values so obtained was as accurate as using the equation in the form $V_M = \frac{v}{s} K_m + v$ and calculating statistically the value of K_m, which corresponds to the negative of the slope of the line when v is plotted against v/s. Thus calculated the K_m value was $7{\cdot}19 \times 10^{-2}$ (g. azocasein/100 ml. reaction mixture).

Calliphora larval gut proteinase is partially inactivated after storage for 24 hr. at 37° and 44°, but for short periods of incubation (2 hr.) the maximum activity was at 44°. For an incubation period of 12 hr. there was a fourfold decrease in activity, during the final 2 hr. period, at 44° compared with that at 25°.

Over the range 5° to 35° the temperature-activity relationship follows that given by the Van't Hoff–Arrhenius equation, $\frac{d \ln K}{dT} = \frac{E_A}{RT^2}$. The activation energy, calculated to be 13 060 cal./mole, falls within the range of values obtained for trypsin and chymotrypsin (Butler, 1941) and for the tryptic digestion of casein (Sizer & Josephson, 1942).

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The Sensitivity to pH of the Inhibitory Effects of Dinitrophenol on Squid Giant Axons. By P. C. Caldwell.* (*Laboratory of the Marine Biological Association, Plymouth*)

During investigations of the relationship between the active extrusion of sodium and metabolism in giant axons of the squid *Loligo forbesi* (Hodgkin & Keynes, 1955; Caldwell, 1956*a*; Caldwell & Keynes, 1957) it has been found that certain inhibitory effects of 2:4-dinitrophenol (DNP) are sensitive to the pH of the bathing solution. When axons were bathed in sea water of pH about 8 containing 0·2 mM-DNP, the sodium extrusion was practically unaffected and paper chromatography showed that the level of ATP in the axoplasm was not greatly affected, although most of the arginine phosphate broke down. However, after 60–95 min. soaking in sea water of pH 6·5–6·8 containing 0·2 mM-DNP, the sodium extrusion was markedly inhibited and both the ATP and the arginine phosphate broke down. The effects of 0·2 mM-DNP on the sodium extrusion process and the metabolism of the axons are therefore much more marked at pH 6·5–6·8 than at pH 8. Also it seems that the sodium

* Beit Memorial Fellow.

extrusion is connected with the presence of ATP in the axons.

Analyses of the DNP taken up by the axons under these conditions have shown that at pH 6·5–6·8 the axoplasm contains about 0·29 mM-DNP/kg. (mean of 5 determinations; range 0·25–0·35), while at pH 8·0–8·2 it contains about 0·04 mM-DNP/kg. (mean of 4 determinations; range 0·03–0·07). This suggests that the effects of pH are due to an effect on the ease with which DNP penetrates into the axons. It seems likely that the axon membrane can be penetrated by the undissociated form of DNP, the amount of which decreases with increasing pH, but not by the negatively charged dissociated form. In these circumstances $\log_{10}$ (DNP concentration inside)/(DNP concentration outside) would be approximately equal to (internal pH − external pH). The amounts of DNP taken up indicate that the pH in the axons should be in the range 6·6–7·6 and this is to be compared with measurements with glass electrodes (Caldwell, 1954, 1955, 1956*b*) which have given values in the region of 7.

In these experiments the axons remained excitable until taken for analysis. The results emphasize the importance of controlling the pH in work in which DNP is used as an inhibitor.

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Comparative Studies on the Liver Sulphatases. By A. B. Roy. (*Department of Biochemistry, University of Edinburgh*)

Although the arylsulphatases are of widespread occurrence there is little quantitative information available on their distribution, due to the many difficulties inherent in their determination in unpurified preparations. A method has been devised for the assay of the sulphatases in liver, based on the isolation of the various cell fractions from a sucrose homogenate of the tissue. The sulphatase activities of each fraction are then determined and the sum of these activities taken as the total activity of the tissue. Arylsulphatases A and B are together determined using nitrocatechol sulphate as substrate (Roy, 1953) and arylsulphatase C using *p*-nitrophenyl sulphate (Roy, 1956*b*). All assays are carried out under conditions optimal for the species in question as there are considerable species variations between these conditions. Steroid sulphatase and the steroid sulphate synthesizing system were determined by the methods of Roy (1957) and Roy (1956*a*) respectively.

Four samples from one rat liver were assayed by the above method and gave a mean value for the sulphatase A+B activity of 213 units/g., with a range of 196–238 units/g., and for the sulphatase C activity of 20·5 units/g., with a range of 19·2–21·7 units/g. One unit of sulphatase activity is taken as the amount of enzyme which will hydrolyse 1 μM of sulphate ester per hour under the appropriate optimal conditions. These results show the method to be a satisfactory one for the assay of the sulphatases in liver.

The method has been applied to the study of the sulphatases in the livers of various species. The results show that there are important quantitative differences between the sulphatase activities in different species. Arylsulphatases of the A and B type have been detected in all the species studied but sulphatase C is apparently lacking from the livers of the guinea-pig and the frog. Steroid sulphatase is lacking from these two species and from the fowl. The intracellular distribution of the sulphatases in all the species studied is similar, apart from minor quantitative differences, to that in the rat. There are also large quantitative differences in the amounts of the steroid sulphate synthesizing system present in the various species. The effects of various treatments on the levels of sulphatase activity will be discussed.

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Specific Lipopolysaccharides of *Haemophilus bronchisepticus*, *H. pertussis* and *H. parapertussis*. By A. P. MACLENNAN (introduced by D. A. L. DAVIES). (*Microbiological Research Establishment, Porton, Wiltshire*)

Lipopolysaccharides were extracted from acetone-dried cells of *Haemophilus bronchisepticus*, *H. pertussis* (Phase I) and *H. parapertussis* with 45% phenol (Westphal, Lüderitz & Bister, 1952) and separated from nucleic acid by differential ultracentrifugation. The preparations were strongly pyrogenic in rabbits, but only the *H. bronchisepticus* lipopolysaccharide was significantly toxic for mice and rabbits. Each substance precipitated at high dilution with homologous bacterial cell antisera and when examined by the agar diffusion precipitin method (Ouchterlony, 1953; Crumpton & Davies, 1956) gave a single line of precipitation with these homologous antisera. As little as 50 μg. of *H. bronchisepticus* lipopolysaccharide gave a visible reaction on diffusion plates.

Paper chromatography of acid hydrolysates showed that each of the substances contained glucosamine and an aldoheptose, possibly D-*glycero*-L-*manno*heptose. The H_2SO_4-cysteine reaction (Dische, 1953) revealed approximately 20% aldoheptose in the *H. bronchisepticus* and *H. parapertussis* lipopolysaccharides and 40% in the *H. pertussis* preparation, measured as D-*glycero*-L-*manno*heptose. A small amount of hexose was also detected by this method. The *H. bronchisepticus* preparation after acetone fractionation was only slightly heterogeneous in the analytical ultracentrifuge (N, 7·7%; P, 2·2%; glucosamine, 16·5%; aldoheptose, 22%; hexose, 5% as glucose; ash, 7·2%; $[\alpha]_D^{18} - 33{\cdot}8$). The high N value could not be accounted for as glucosamine or α-amino N. Hydrolysis with either 1% (v/v) or 15% acetic acid for 4 hr. at 100° liberated a water-insoluble material (20%) which was partly composed of a chloroform-soluble lipid and another lipid soluble in ether.

Twenty NCTC strains of *H. bronchisepticus*, colonially alike, were shown by agar diffusion precipitin analysis to possess at least eight antigens in common. In addition, a line of precipitation formed by only thirteen of the otherwise antigenically homogeneous strains was identical with the single line given by the purified lipopolysaccharide. Acetone dried cells of strains not forming the lipopolysaccharide line were unable to absorb antibody to lipopolysaccharide from antisera, and live cells of these strains did not elicit production of this antibody when injected into rabbits. Only those strains possessing the lipopolysaccharide killed mice on intraperitoneal or intranasal injection.

I thank Dr Jean Horton for *H. pertussis* cells and Mr D. C. Hawkins for technical assistance.

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The Iron Content of Human Tissues. By LYNDA M. H. KERR. (*Rheumatic Unit, Northern General Hospital, Edinburgh*)

As part of an investigation into the anaemia associated with rheumatoid arthritis, a study is being made of the iron content of the body tissues. It was therefore necessary to have available results from control material for comparison. For this, tissue has been obtained at forty-four autopsies and the iron content determined by the dipyridyl method of Ramsay & Campbell (1954). The causes of death include sudden traumatic injury, acute vascular episodes, acute and chronic infection, carcinoma, polycythemia vera and haemosiderosis.

As some of the material was available only after fixation in formalin, a comparison has been made of the results obtained from the analysis of ten different samples of tissues both fresh and after fixation in formol saline for 2–4 weeks. No significant difference in the iron content was found between the two groups.

The average values for the iron content of a wide range of tissues will be presented together with a discussion of the effect of sex, age and disease on these values. Although liver and spleen contain by far the greatest amount of iron (15 and 22 mg./100 g. wet weight of tissue respectively), significant amounts of iron were found in all tissues examined. A slight increase in the iron content of liver and spleen (28 and 47 mg./100 g. respectively) was found in polycythemia vera, while grossly increased amounts occurred in haemosiderosis, the values being of the order of 400 and 200 mg./100 g. respectively.

Analysis of the results suggest that less iron is stored by the female than by the male. Only in the case of spleen is there a significant relationship between age and iron content. It is, however, difficult to assess the influence of disease on these results.

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The Fermentation of DL-[2-^{14}C]Lactate by the Rumen Organism LC. By J. N. LADD (introduced by S. R. ELSDEN). (*A.R.C. Unit of Microbiology, Department of Microbiology, The University, Sheffield* 10)

The rumen organism LC (Elsden, Volcani, Gilchrist & Lewis, 1956) ferments DL-lactate to H_2, CO_2, acetate, propionate, *n*-butyrate, *n*-valerate and small amounts of *n*-hexanoate. The fermentation of DL-[2-^{14}C]lactate by washed suspensions of LC has now been investigated. The fatty acids formed were separated by the chromatographic method of Bueding & Yale (1951) and degraded stepwise by the procedure of Phares (1951).

The CO_2 produced was unlabelled. The acetate formed was carboxyl-labelled and the propionate was labelled *exclusively* on the methylene position. The specific activity of the butyrate was twice that of the butyrate carboxyl; the specific activity of valerate was twice that of the valerate carboxyl and the specific activity of caproate was three times that of the caproate carboxyl.

If propionate were formed by the decarboxylation of succinate as in *Veillonella gazogenes* (Johns, 1951) then two forms of labelled propionate,

$$CH_3 \cdot {}^{14}CH_2 \cdot CO_2H \quad \text{and} \quad {}^{14}CH_3 \cdot CH_2 \cdot CO_2H$$

would be produced, which together would degrade as $^{14}CH_3 \cdot {}^{14}CH_2 \cdot CO_2H$ (Leaver, Wood & Stjernholm, 1955). Further, the propionate formed when lactate is fermented in the presence of $^{14}CO_2$ would be carboxyl-labelled and the specific activity of the carboxyl group would approximate to that of the $^{14}CO_2$. When lactate was fermented under these conditions, the specific activity of the propionate carboxyl was only 6% of that of the $^{14}CO_2$. It is concluded that propionate is not formed by the succinate pathway. Cell-free extracts of LC catalyse an exchange reaction between pyruvate carboxyl and CO_2 and this may account for the slight labelling of the propionate.

The formation of fatty acids from lactate by LC may be explained by the following equations:

$$CH_3 \cdot {}^{14}CHOH \cdot CO_2H + 2(H) \rightarrow CH_3 \cdot {}^{14}CH_2 \cdot CO_2H$$

$$CH_3 \cdot {}^{14}CHOH \cdot CO_2H \rightarrow CH_3 \cdot {}^{14}CO_2H + CO_2 + 4(H)$$

$$2CH_3 \cdot {}^{14}CO_2H + 4(H) \rightarrow CH_3 \cdot {}^{14}CH_2 \cdot CH_2 \cdot {}^{14}CO_2H$$

$$CH_3 \cdot {}^{14}CH_2 \cdot CO_2H + CH_3 \cdot {}^{14}CO_2H + 4(H) \rightarrow CH_3 \cdot {}^{14}CH_2 \cdot CH_2 \cdot CH_2 \cdot {}^{14}CO_2H$$

$$3CH_3 \cdot {}^{14}CO_2H + 8(H) \rightarrow CH_3 \cdot {}^{14}CH_2 \cdot CH_2 \cdot {}^{14}CH_2 \cdot CH_2 \cdot {}^{14}CO_2H$$

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The Purification and Properties of the L-Threonine Deaminase of the Rumen Micro-organism LC1. By D. J. WALKER (introduced by S. R. ELSDEN). (*A.R.C. Unit for Microbiology, Department of Microbiology, The University, Sheffield* 10)

The rumen organism LC (Elsden, Volcani, Gilchrist & Lewis, 1956) was shown by Lewis & Elsden (1955) to ferment threonine and serine with the production of ammonia, hydrogen, carbon dioxide and volatile fatty acids.

The L-threonine deaminase has been purified as follows: 1 part of vacuum-dried cells was extracted for 1 hr. at 37° with 10 parts of 0·01M-phosphate buffer (KH_2PO_4–Na_2HPO_4), pH 7·8. Nucleic acids were precipitated from the extract by treatment with protamine sulphate. The supernatant was brought to 70% saturation with solid $(NH_4)_2SO_4$, and the precipitate, which contained the enzyme, centrifuged off. The precipitate was suspended in a small volume of 70% saturated $(NH_4)_2SO_4$ solution and concentration gradient elution with ammonium sulphate carried out (Schwimmer, 1953; Sayne & Greenberg, 1956).

Further purification was achieved by pooling the fractions containing the enzyme, precipitating the protein by addition of solid $(NH_4)_2SO_4$, redissolving the precipitate in 0·01M-phosphate buffer pH 7·8 and chromatography on a calcium phosphate gel-cellulose column. By this means, a preparation with a specific activity of 500 units/mg. protein was obtained: the specific activity of the crude extract was 5 units/mg. protein (1 unit = the amount of enzyme necessary to catalyse the formation of 1 μmole of oxo acid from L-threonine in 5 min. under standard conditions).

The optimum pH for threonine deamination was in the range 9·0–10·0. The enzyme was specific for L-threonine, and the presence of the D-isomer inhibited the deamination of L-threonine. Serine deaminase activity could not be separated from L-threonine deaminase activity. The K_m values are $6{\cdot}8 \times 10^{-3}$M and $3{\cdot}0 \times 10^{-3}$M for L-serine and L-threonine respectively.

On freeze-drying enzyme preparations in the presence of glutathione and $(NH_4)_2SO_4$, enzyme activity was almost completely lost, but could be restored by adding pyridoxal phosphate; a further but slight increase in activity was obtained if glutathione was also added. The K_m for the apoenzyme-pyridoxal phosphate system was $1{\cdot}8 \times 10^{-6}$ M.

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The Structure of Muramic Acid. By L. H. KENT. (*Microbiological Research Establishment, Porton, Wiltshire*)

Muramic acid (Work, 1957), which occurs in 6N-HCl hydrolysates of 'spore peptide' (Strange & Powell, 1954) and bacterial cell walls (Cummins & Harris, 1956) has been assigned a provisional structure as a 3-*O*-α-carboxyethylhexosamine (Strange, 1956). The green colour given by muramic acid after treatment with borate buffer in the Bial test suggested the probability that the hexosamine was D-glucosamine and the synthesis of 3-*O*-α-carboxyethylglucosamine was, therefore, attempted. *N*-Acetyl-4:6-benzylidene-α-methylglucosaminide (Neuberger, 1941) was converted into its 3-*O* sodio derivative by means of sodium hydride and this was then condensed in boiling anhydrous tetrahydrofuran with ethyl α-DL-iodo-orthopropionate. The product, after hydrolysis with 6N-HCl for 2 hr., was freed from glucosamine by means of IR 120 (buffered to pH 6·05) and was eluted as a sharp peak from a column of Dowex 50 by 0·3N-HCl (Strange & Dark, 1956). The terminal portion of the eluate was treated with IR-4B (OH form) to remove HCl and the dried product recrystallized from 90% (v/v) ethanol–H_2O. The crystals under microscopic examination appeared identical with muramic acid. The synthetic compound gave a maximum absorption in the Elson and Morgan reaction at 510–520 mμ, characteristic of muramic acid, in contrast to glucosamine and galactosamine which give maxima between 530 and 535 mμ, and it behaved identically with the natural material on paper chromatography in phenol/H_2O and *tert.*-butanol/H_2O.

These results strongly support the proposal that muramic acid is 3-*O*-α-carboxyethyl-D-glucosamine but there is still ambiguity concerning the configuration about the central C atom of the lactic acid moiety since this is asymmetric. The stability of muramic acid to boiling HCl and its possession of only four groups capable of being acetylated differentiates it sharply from neuraminic acid, despite the fact that both compounds are amino carboxylic acids derived from glucosamine carrying a chain of three additional C atoms. Nevertheless, the two structures are formally very similar.

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The Water-soluble Carbohydrates of the Onion, *Allium cepa* L. By J. S. D. BACON. (*Department of Biochemistry, Macaulay Institute for Soil Research, Aberdeen*)

The water-soluble carbohydrates of the onion bulb consist of fructose, glucose, sucrose and a series of oligosaccharides (Dr J. Edelman, personal communication, 1951). Paper chromatograms suggest that the chain-length of the oligosaccharides does not exceed eight; there is no evidence for the presence of fructose polysaccharides like those found in many other monocotyledons (cf. Archbold, 1940). The oligosaccharides are mainly non-reducing; they are converted to a mixture of glucose and fructose by the action of yeast invertase, an indication that they consist chiefly of β-fructofuranose residues.

Chromatography on charcoal reveals that each oligosaccharide spot on paper chromatograms corresponds to two or more substances. The two major trisaccharides have been isolated from boiling-water extracts of the bulbs by gradient elution from charcoal-Celite (Bacon & Bell, 1953)

and partition chromatography with aqueous *n*-butanol on Celite (Lemieux, Bishop & Pelletier, 1956), and identified as 1^F-β-fructosylsucrose (crystalline) and 6^G-β-fructosylsucrose (amorphous) by their optical rotations, infra-red absorption spectra, and other properties. The former is known to be a normal constituent of plant tissues (*Helianthus tuberosus*: Dedonder, 1952; Allen & Bacon, 1956), but the latter has been found hitherto only among the products of invertase action (Gross, Blanchard & Bell, 1954) and is of interest because it could serve as a basis for the synthesis of fructosans with glucose residues within the chain (cf. Schlubach & Lübbers, 1956). There was no sign of 6^F-β-fructosylsucrose ('kestose': Albon, Bell, Blanchard, Gross & Rundell, 1953), nor of raffinose, in the extracts.

The oligosaccharides are distributed unevenly in the mature onion bulbs, being absent from the outer scales, while in the inner ones they may constitute more than half the soluble carbohydrate. There is more oligosaccharide at the base of the scales than at their tops and considerable variations occur from point to point round the circumference of each scale.

Vacuum infiltration of glucose into small pieces of bulb scales leads in 1–3 days to little or no change in the free fructose, but to moderate increases (50–100%) in the ketose contents of the di-, tri-, and tetra-saccharide fractions.

This work was done with the technical assistance of Miss A. M. Joss and Miss D. M. Stewart. I wish to thank Dr V. C. Farmer for examining the infrared absorption of the trisaccharides.

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The Occurrence of β-Glucuronidase, β-Glucosidase, β-Galactosidase and α-Arabinosidase in Insects. By D. Robinson. (*Department of Biochemistry, St Mary's Hospital Medical School, Paddington, London, W. 2*)

The assay of β-glucuronidase (Mead, Smith & Williams, 1955), and β-glucosidase (Robinson, 1956), using fluorigenic substrates, has been described. The aglycone, 4-methylumbelliferone is intensely fluorescent in alkaline solution, while the glycosides of this substance have virtually no fluorescence under the same conditions. Two new glycosides, the β-galactoside, and the α-arabinoside of 4-methylumbelliferone have now been synthesized and used for enzyme assay.

It was shown (Robinson, 1956) that β-glucuronidase and β-glucosidase are widespread in insect tissues. A further survey of some economically important insects has revealed a marked ability to hydrolyse glycosides related configurationally to β-D-glucose.

The substrate concentrations used were: β-glucuronide 1×10^{-4}M, β-glucoside 5×10^{-4}M, β-galactoside 9×10^{-4}M, α-arabinoside 1×10^{-2}M, the last two concentrations being close to saturation point. Optimum pH's varied with the species between 4·2 and 5·5 for each enzyme. For routine assay the following values were chosen; for β-glucuronidase pH 4·4, for other glycosidases pH 5·0.

The four enzymes studied occurred in all species examined, but in different relative proportions. Little variation was found between individual insects of any one species.

A partial separation of the β-galactosidase activity of locust-crop fluid from the β-glucosidase in the same material has been achieved by paper electrophoresis.

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The Metabolism of *m*-Dinitro-[^{14}C]benzene in the Rabbit. By D. V. PARKE and R. T. WILLIAMS. (*Department of Biochemistry, St Mary's Hospital Medical School, London, W. 2*)

m-Dinitrobenzene is of industrial importance as an intermediate for dystuffs and as a constituent of mining explosives. It is an acute poison and produces methaemoglobinemia, anaemia, liver damage, cerebral paralysis and convulsions (Kiese, 1949). Previous workers have shown that it is reduced *in vivo* to *m*-nitroaniline (Beloborodova, 1945) and *in vitro* to *m*-nitrophenylhydroxylamine (Lipschitz & Osterroth, 1924).

Using ^{14}C-labelled *m*-dinitrobenzene we have shown that when administered orally to rabbits at dose levels of 80–100 mg./kg., 90 % of the dose is excreted in the urine in two days, as *m*-nitraniline and/or *m*-phenylenediamine (35 %), 2-amino-4-nitrophenol (15 %), 4-amino-2-nitrophenol (2 %) and 2:4-diaminophenol (40 %). Traces of unchanged *m*-dinitrobenzene (< 0·2 %) and 3:3′-dinitroazoxybenzene are also present, but 2:4-, 2:6-, and 3:5-dinitrophenols are absent. Since all the metabolites were estimated after acid hydrolysis of the urines the 4-amino-2-nitrophenol could be an artifact derived from *m*-nitrophenylhydroxylamine. The 3:3′-dinitroazoxybenzene is also probably an artifact from the same source.

Nitroaniline, m.p. and mixed m.p. 112°, equivalent to 9 % of the dose, and a trace of 3:3′-dinitroazoxybenzene, m.p. and mixed m.p. 143°, were isolated from the non-hydrolysed urine. 2-Amino-4-nitrophenol, m.p. and mixed m.p. 142–143° equivalent to 5 % of the dose was isolated from the acid-hydrolysed urine.

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The Fate of 4:6-Dinitro-*o*-cresol (DNOC) and some Related Compounds in the Locust, *Schistocerca gregaria*. By T. KIKAL and J. N. SMITH. (*Department of Biochemistry, St Mary's Hospital Medical School, London, W. 2*)

DNOC has been administered to locusts, and extracts of excreta and intestine separated on paper chromatograms. Examination of the absorption spectra of eluted zones, and R_F values of eluates in a number of solvents suggests that at sublethal doses, DNOC is partly converted to the relatively non-toxic 6-acetamido-4-nitro-*o*-cresol.

When 6-acetamido-4-nitro-*o*-cresol is administered to immature adult *Schistocerca gregaria* it is excreted unchanged, whereas if 6-amino-4-nitro-*o*-cresol is given, substantial amounts of the acetylated metabolite are found (cf. Myers & Smith, 1953). Small amounts of the β-glucoside and ethereal sulphate of 6-amino-4-nitro-*o*-cresol have now been identified by their spectroscopic, chromatographic, and ionophoretic properties as metabolites of this compound in *Schistocerca gregaria*.

These *O*-conjugates are more readily detected in extracts of intestine and malpighian tubules than in excreta and it seems probable that they are hydrolysed, after excretion into the midgut, by the active β-glucosidase and arylsulphatase found there. These enzymes may be the same as those occurring in the crop fluid of locusts (Robinson, Smith & Williams, 1953; Robinson, 1956).

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The Use of Long-chain Quaternary Ammonium Compounds in the Fractionation of Heparin. By J. E. SCOTT,* S. GARDELL and I. M. NILSSON. (*Chemistry Department II, Karolinska Institutet, Stockholm, Sweden*)

The reaction of long-chain quaternary ammonium salts with polymeric anions resulting in the formation of very insoluble precipitates has been previously described (Jones, 1953; Scott, 1955*a*, *b*, 1956*a*). It has been shown that the solubility of these complexes in salt solutions is determined by (1) the type of acid group, (2) the density of charge, and (3) the molecular weight, of the polyanion (Scott, 1956*b*).

Since the biological activity of heparin may be dependent on the same factors it seemed of interest to attempt to fractionate heparin by this technique.

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Crude heparin of low activity (<40 units/mg.) was dissolved in 1·5N-KCl solution containing an excess (about 5 mg./mg. heparin) of cetylpyridinium chloride. Water was added to reduce the concentration of KCl in stages to 1N. The precipitate formed in each stage was removed, dissolved in 33% saturated saline, treated with Fuller's earth to remove cetylpyridinium, dialysed, concentrated in vacuum and freeze-dried. The 1N-KCl supernatant solution was also treated with fuller's earth, dialysed and freeze-dried. Anticoagulant potency of the fractions was assayed by the methods of Magnusson & Nilsson (1957), using human plasma and bovine plasma. Sulphate estimations were performed by a gravimetric barium sulphate method.

Six or more fractions were dealt with in experiments using several commercial crude bovine lung heparin preparations. The fractions which were soluble only in the highest concentrations of KCl (>1·35N) as cetylpyridinium complexes, were found to have the highest activity. In the antithrombin and recalcification tests the activity of these fractions ranged between 150 and 200 units/mg. In the whole blood system, however, lower activities were found (about 140 units/mg.). Activity was small and sometimes lacking in fractions soluble in less than 1N-KCl.

In one experiment the material forming cetylpyridinium complexes insoluble at higher concentrations than 1N-KCl (F1) had 9·2% S, the fraction soluble in 1N-KCl but insoluble in 0·6N-KCl (F2) had 6·3% S. The remainder, soluble in 0·6N-KCl (F3) had 1·2% S. This last fraction, which in some cases actually increased the clotting rate of recalcified plasma, is under further investigation.

F1 contained glucosamine, F2 glucosamine with a little galactosamine, and F3 considerable quantities of galactosamine with glucosamine. (Method of Gardell, 1953.)

These experiments demonstrate the correlation between anticoagulant potency and the concentration of salt solution in which the cetylpyridinium complexes of 'heparins' are soluble.

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Purification of an Enzyme Converting Porphobilinogen to Uroporphyrin. By W. H. Lockwood and C. Rimington. (*Department of Chemical Pathology, University College Hospital Medical School, London, W.C.* 1)

Haemolysates of human and chicken erythrocytes convert porphobilinogen to uro- copro- and protoporphyrins (all series III isomers). By mild heating the system is changed so that only uroporphyrin I is produced (Booij & Rimington, 1957). The enzyme converting porphobilinogen to uroporphyrin III has been purified. Activity was estimated as μg. uroporphyrin/ml./hr. at pH 8·4 and 37°.

Centrifuged haemolysate of chicken erythrocytes (100 ml., protein content 12%, enzyme activity 3·25 μg./ml./hr.) was allowed to stand at room temperature with 20 ml. of chloroform for 5 hr. with occasional shaking. The bulky precipitate of haemoglobin was centrifuged off, leaving 82 ml. of solution (activity 3·34 μg./ml./hr.). This was dialysed overnight against running tap water giving 87 ml. (activity 3·02 μg./ml./hr.). Calcium phosphate gel (2 ml.) was added. The supernatant after centrifuging had an activity of 0·37 μg./ml./hr. The gel was extracted three times with 0·1M-phosphate buffer, pH 8, to give 2·1 ml. of extract (activity 133 μg./ml./hr., protein content 0·9%). Based on protein, this represents a 500-fold purification. The eluate may be stored at 4° under benzene and is stable for several weeks. It can also be stored as dry acetone powder; re-extraction with water yields a solution with 90% of original activity.

The final solution is straw-coloured and contains only a faint trace of haemoglobin. It produces uroporphyrin III from porphobilinogen; paper chromatography shows no uroporphyrin I. The pH optimum lies between 8 and 8·5. At pH 7 the enzyme still has about 50% of its maximum activity. If heated for 15 min. at 55°, it produces from porphobilinogen at 37° uroporphyrin I with a trace of uroporphyrin III. No ether-soluble porphyrin is formed by either the heated or unheated enzyme. We suggest the name, *porphobilinogenase*.

We thank Miss A. Benson for carrying out paper chromatography on the uroporphyrin and one of us (W.H.L.) thanks the Nuffield Foundation for a Nuffield Dominion Travelling Fellowship and the Australian National Health and Medical Research Council for a grant.

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An Enzyme Defect in Human Neonatal Jaundice and in Gunn's Strain of Jaundiced Rats. By G. H. LATHE and MARJORIE WALKER. (*Bernhard Baron Memorial Research Laboratories, Queen Charlotte's Maternity Hospital, London, W. 6, and the Institute of Obstetrics and Gynaecology, University of London*)

Normal rat-liver slices incubated in Ringer-phosphate-bicarbonate medium containing plasma proteins conjugated bilirubin with glucuronic acid (as shown by the 'direct' van den Bergh reaction). Maximal conjugation occurred at 16 mg. bilirubin per 100 ml. of medium, containing 20% plasma. Liver slices of adult rat, rabbit, and monkey (*Macaca mulatta*) conjugated about 55 μg. bilirubin/g. wet liver/hr. The guinea pig conjugated 17 μg./g. wet liver/hr.

Liver slices from homozygous females of Gunn's strain of jaundiced rats (Gunn, 1944) did not convert bilirubin to direct reacting pigment, as predicted by Malloy & Lowenstein (1940). Liver slices from two newborn human infants, taken within minutes of death, did not conjugate, as was suggested from studies of plasma bile pigments by Billing, Cole & Lathe (1953). Oxygen consumption was maintained in these specimens, and control rat livers subjected to similar postmortem treatment maintained their ability to conjugate.

Rat-liver suspensions (10%) showed maximal bilirubin conjugation in the presence of crude uridine diphosphate glucuronic acid (UPDGA) of Dutton & Storey (1954) at 24 mg. bilirubin/100 ml. medium containing 24% plasma. The rate of conjugation was 0·4 mg./g. wet liver/hr., which is about half the maximal *in vivo* excretion of bilirubin by the rat (Weinbren & Billing, 1956). Similar rates of conjugation were obtained with liver suspensions from adult and baby mice, and adult rabbits. Guinea pigs conjugated less. In the absence of added UPDGA concentrates, conjugation by rat-liver suspensions was about 40% of that found in fortified preparations. In the presence of added UPDGA activity was greatest in the microsome fraction.

Liver suspensions from human newborn infants and from homozygous Gunn rats did not conjugate bilirubin even in the presence of UPDGA. Since Gunn rat-liver slices conjugated *o*-aminophenol, the production of UPDGA was normal. By analogy with other glucuronic acid conjugating systems (Dutton, 1956) it is suggested that in the newborn infant and in Gunn's rats the enzyme transferring the glucuronyl radical to bilirubin is absent or deficient.

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The *N*-Terminal Groups and Partial Fractionation of Thymus Histones. By D. M. PHILLIPS (*Chester Beatty Research Institute, Institute of Cancer Research, Royal Cancer Hospital, Fulham Road, London, S.W. 3*)

Using the fluorodinitrobenzene method, the *N*-terminal groups of calf thymus histones were found to be; proline 40–45%, alanine 35–40%, serine and lysine about 5% each, and smaller amounts of eight other amino acids. These values are expressed as the percentage of the total *N*-terminal groups found, which amounted to approximately 1 mole per 25000 weight of histones, after due corrections had been made. The dinitrophenyl histones were hydrolysed for 4·5 hr. at 100° in 11N-HCl. The estimations were made with two-dimensional chromatograms on no. 7 Whatman paper buffered with pH 6 phthalate buffer (Blackburn & Lowther, 1951). The first solvent was *sec.*-butanol (or *tert.*-pentanol) saturated with the buffer and the second was 1·5M-phosphate buffer, pH 6 (Levy, 1954). Proline was identified by the presence of strong spots of the two open-chain dinitrophenyl derivatives formed from dinitrophenyl proline on hydrolysis (Scanes & Tozer, 1956).

Sodium chloride-ammonia fractionation of the histones (Davison & Shooter, 1956) showed that those precipitated are relatively richer in arginine, whereas those remaining in solution are richer in lysine. It has been found that the former have mainly alanine end-groups (about 70% of the total end-groups) and the latter mainly (also about 70%) proline end-groups. Partial fractionations have also been achieved on alumina columns with gradients of potassium phosphate. The main peak

isolable from such columns consists of lysine-rich histones, and about 75% of the end-groups are proline residues. Such material has been further fractionated on carboxymethylcellulose columns (Peterson & Sober, 1956) with a sodium acetate-sodium chloride gradient (Davison, 1957) to give three fractions, the chief of which had approximately 85% proline end-groups. The small fraction of the histones (amounting to about 2% of the total), which is extractable from deoxyribonucleoprotein by citric acid (Davison & Butler, 1954) was found to have valine as its chief *N*-terminal group.

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The Detection of 4:5-Diaminouracil as a Metabolite of *Eremothecium ashbyii*. By T. W. Goodwin and D. H. Treble. (*Department of Biochemistry, The University, Liverpool* 3)

The stimulatory effect of purines on flavinogenesis by *Eremothecium ashbyii*, together with results of experiments using labelled adenine and serine, suggest that a purine (or its riboside or ribotide) is a normal precursor of riboflavin in this organism (Goodwin & Pendlington, 1954; McNutt, 1956; Goodwin & Jones, 1956). A possible intermediate is 4:5-diaminouracil formed by loss of the $C_{(8)}$ of xanthine. It is impossible to detect 4:5-diaminouracil as such because of its instability, but it might be trapped as 2:4-dihydroxy-6:7-dimethylpteridine (6:7-dimethyllumazine) by adding diacetyl to culture media, for 4:5-diaminouracil and diacetyl readily condense in this way (Birch & Moye, 1957). Using diacetyl as trapping agent, a mixture of compounds was obtained; these were not observed in uninoculated medium treated with diacetyl. The mixture proved difficult to resolve and better results were obtained using glyoxal as the trapping agent; in this case the product should be lumazine.

In a typical experiment, glyoxal was added to 48 hr. cultures (5 l.) to give a final concn. of 50 mg./100 ml. After 5 days the cells were harvested and the medium evaporated to dryness *in vacuo*. The combined residue and cells were extracted several times with 100 ml. portions of ethanol containing 1% (v/v) NH_3 (0·880). The combined extracts were reduced to small volume *in vacuo* and chromatographed on Whatman no. 3 paper: solvent butanol/glacial acetic acid/H_2O (4:1:5 by vol.). The region of the chromatogram (R_F about 0·33) which exhibited in ultraviolet light a weak blue fluorescence, greatly enhanced in the presence of NH_3, was eluted with 50% (v/v) aqueous methanol containing 1% (v/v) NH_3 (0·880) and rechromatographed using propanol/NH_3 (0·880)/H_2O (6:3:1 by vol.) as solvent. Two fractions were obtained: *A* the smaller fraction (R_F 0·25) and *B* (R_F 0·60). After further purification, *A* was shown to be lumazine by mixed chromatography with an authentic specimen in three solvent systems, the two detailed above and butanol/ethanol/H_2O (50:15:35 by vol.). The absorption spectra at pH 6·5 and 12·5 of the isolated compound and authentic lumazine, were identical. *A* could easily be separated chromatographically from 6:7-dimethyllumazine which has an absorption spectrum similar to that of lumazine.

These observations indicate that 4:5-diaminouracil is a normal metabolite of *E. ashbyii*.

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Properties of Lucerne and other Saponins. By C. B. Coulson. (*Department of Agricultural Chemistry, University College of North Wales, Bangor*)

The saponin fraction of lucerne (*Medicago sativa* L.) is claimed to have growth-inhibiting properties for chicks (Peterson, 1950; Heywang & Bird, 1954). The sapogenins apparently have no growth depressant effect (Potter & Kummerow, 1954). Lindahl, Cook, Davis & Maclay (1954) reported the production of bloat in ruminants by this fraction, although Ferguson & Terry (1956) were unable to confirm this. Both the saponin fraction from lucerne and ladino clover (*Trifolium repens* r. *giganteum*) inhibit peristalsis of isolated rabbit intestine, or rumen strips (Walter, Van Atta, Thompson & Maclay, 1954; Walter, Bickoff, Thompson, Robinson & Djerassi, 1955; Wilson, Sideman & De Eds, 1956).

In the present work, lucerne (whole plant) gave a yield of 0·6 % dry weight as crude water-soluble saponin; from white clover (*T. repens* S 100) 0·04 % dry weight of the poorly soluble non-haemolytic saponin was obtained. A comparison of the lucerne saponins with quillaja saponins (British Drug Houses, Ltd.) showed that although their haemolytic activity and foaming properties were similar, the lucerne saponins were much less active on smooth muscle (rabbit). Moreover, this inhibition was not reversed by a dilute cholesterol suspension in dilute aqueous Tween 80.

Higher quantities of commercial quillaja saponin (2–3 %) are required for a significant depression of rat growth than has been reported for the chick (Peterson, 1950). Linoleic esters (segregated sunflower seed oil, kindly supplied by Messrs H. M. Faure and Co. Ltd.), but not free linoleic acid (Parker, Koos & Swern, 1955), produced a partial reversal of the growth depression in the rat due to both saponin and cholesterol, and possibly permits some cholesterol reversal of the saponin depression.

X-ray studies on rats show that the commercial quillaja saponin does not produce rachitic bone formation, nor does it prevent the cure of rats fed a rachitic diet when fed at a 2 % level.

Ascending paper chromatography, using a borate buffered paper with *n*-butanol/ethanol/pH 9 borate buffer [made from 500 ml. solution A (0·067 M-boric acid and 0·067 M-KCl) and 213 ml. solution B (0·067 M-NaOH)] and detecting by the Carr–Price reagent, shows two major and two minor constituents in lucerne saponin with smaller quantities of three other constituents. Multiple development with ethyl acetate/acetic acid/water (13:3:3 by vol.) as the solvent confirms this. Paper electrophoresis using borate buffer (9·54 g./l. sodium tetraborate) reveals two major and two minor constituents. These methods have been found suitable for other triterpenoidal saponins (Coulson, 1957).

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The Biosynthesis of Leaf Polysaccharides. By P. Andrews* and L. Hough. (*Department of Chemistry, The University, Bristol*)

The polysaccharides of plum leaves (var. Victoria) are constituted mainly of D-galactose, D-glucose, L-arabinose, D-xylose, and D-galacturonic acid with smaller amounts of D-mannose, L-rhamnose, L-fucose, 2-*O*-methyl-D-xylose and glucuronic acid (Andrews & Hough, 1956). The leaf polysaccharides were subjected to a stepwise extraction procedure; each fraction was hydrolysed and the products examined on paper chromatograms. The polysaccharides in the cold water extract resembled the pectic substances and contained galactose, arabinose, galacturonic acid and rhamnose. Hot water extracted further quantities of the pectic polysaccharides, with arabinose as the main monosaccharide component, together with traces of starch and xylan. Larger portions of the leaf starch and xylan were extracted by cold N-NaOH, along with smaller amounts of the pectin components. The hot 2·5 N-NaOH extract contained starch and hemicelluloses composed predominantly of galactose, arabinose, xylose, rhamnose, and galacturonic acid. The total amount of polysaccharides corresponded with the yield (about 100 mg./g. of dried leaf) of monosaccharides obtained on hydrolysis (N-H_2SO_4; 8 hr.; 100°) of methanol-extracted plum leaf and, in each case, the overall proportions of the individual sugars were approximately the same.

The pectin and hemicellulose polysaccharides were labelled during photosynthesis in $^{14}CO_2$ (Hough & Pridham, 1956). This process has been followed by allowing mature plum leaves to photosynthesize in an atmosphere containing initially 5 % of $^{14}CO_2$ for periods from 1 to 48 hr. Incorporation of the $^{14}CO_2$ into the leaves was virtually complete in 6 hr. The leaves from each experiment were exhaustively extracted with methanol and the distribution of the incorporated activity between the residue and the methanol-extract was determined. The activity in the residue, containing the polysaccharides, increased rapidly to 40 % of the

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total after 6 hr., and thereafter to 43% in 24 hr. falling again to 40% in 48 hr.

The distribution of activity among the neutral monosaccharide constituents of the water-soluble leaf polysaccharides was determined by isolating the individual sugars and converting them into characteristic crystalline derivatives, which were then assayed for ^{14}C-activity (as $Ba^{14}CO_3$). The specific activities of galactose, arabinose, rhamnose, and xylose all increased during the first 24 hr., but at different rates, the rate being greatest for galactose and least for xylose. However, during the next 24 hr., the specific activity of arabinose continued to increase, whereas the specific activities of the other monosaccharides decreased.

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PROCEEDINGS OF THE BIOCHEMICAL SOCIETY

The **365th Meeting** *of the Biochemical Society was held in the Department of Biochemistry of Queen's College (University of St Andrews), Dundee, on Friday, 20 September 1957, starting at 10.30 a.m., when the following papers were read:*

COMMUNICATIONS

A Modified Procedure for the Estimation of 'Available Lysine' in Food Proteins. By DOROTHY BRUNO* and K. J. CARPENTER. (*School of Agriculture, Cambridge*)

For many animal products, reaction with fluoro-2:4-dinitrobenzene (FDNB), followed by acid-hydrolysis and ether-washing left solutions in which only ϵ-DNP-lysine appeared to give significant colour (Carpenter & Ellinger, 1955). This measure of lysine related closely to the products' 'gross protein values' (G.P.V.) for chicks in assays where conditions make lysine the usual limiting factor (Carpenter, Ellinger, Munro & Rolfe, 1957). Vegetable materials gave interfering colours in this procedure. A modification uses the reaction of methoxycarbonyl chloride (MC–Cl) with α-amino-groups (Chibnall & Spahr, 1957); with ϵ-DNP-lysine one obtains the ether-soluble (Bailey, 1957):

$$DNP\text{–}NH\cdot(CH_2)_4\cdot CH(COOH)\cdot NH\cdot CO\cdot OCH_3.$$

Ground material is shaken with FDNB as before. We now hydrolyse with 24 ml. 8·1 N-HCl, reflux 16 hr. and make the filtrate to 100 ml. with water. 0·3–1·0 ml. is added to a glass-stoppered tube, extracted twice with 5 ml. di-ethyl ether, and dissolved ether removed by warming. The residue is made alkaline with 2 N-NaOH (after titration of a dummy aliquot plus phenolphthalein) and shaken with 2 ml. of a buffer (8 % w/v $NaHCO_3$ and Na_2CO_3 mixed to give pH 8·5). 0·05 ml. MC–Cl is added with shaking and, after 10 min., 0·75 ml. conc. HCl. Two 5 ml. portions of di-ethyl ether are then shaken in the tube, recovered and evaporated. The residue is redissolved in 10 ml. 1 N-HCl with warming, and read in an absorptiometer (601 filter: 435 mμ). The extinction is compared with those for ϵ-DNP-lysine aliquots carried through the procedure, and the lysine equivalent calculated.

Nine samples of oil-seed, cereal and grass products (Carpenter, Duckworth, Ellinger & Shrimpton, 1955) and eight samples of leaf protein concentrates processed in different ways (Cowlishaw, Eyles, Raymond & Tilley, 1956) gave values from 1·9 to 4·9 g. lysine/16 g. N, which showed a highly significant correlation ($r = 0{\cdot}84$) with their G.P.V. figures. The procedure may therefore prove useful in grading vegetable products. Unfortunately, carbohydrates can increase the loss of DNP-lysine during hydrolysis. Also, chromatography with mixed organic solvents on buffered paper (Blackburn & Lowther, 1951) reveals an interfering band in some cases, though on elution this has shown little extinction at 435 mμ.

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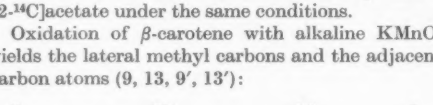
Biosynthesis of β-Carotene from DL-β-Hydroxy-β-methyl-δ-[2-^{14}C]valerolactone by *Phycomyces blakesleeanus* and Carrot Slices. By G. D. BRAITHWAITE and T. W. GOODWIN. (*Department of Biochemistry, The University of Liverpool*)

Growing shake cultures of the fungus *Phycomyces blakesleeanus* will incorporate DL-β-hydroxy-β-methyl-δ-[2-^{14}C]valerolactone (mevalonic acid) into β-carotene. For example, under conditions previously described (Braithwaite & Goodwin, 1957) and assuming that only one isomer is active (Wolf, Hoffman, Aldrich, Skeggs, Wright & Folkers, 1957), addition of $2{\cdot}45 \times 10^6$ c.p.m. (13·6 μmoles) yields β-carotene containing 9600 c.p.m. The efficiency of incorporation is much greater than these figures

indicate because the uptake of lactone from the culture medium is only slight. Many variations of cultural conditions have not overcome this permeability effect. However, even assuming that all the lactone is available, it is still incorporated into β-carotene about ten times more efficiently than is [2-^{14}C]acetate under the same conditions.

Oxidation of β-carotene with alkaline $KMnO_4$ yields the lateral methyl carbons and the adjacent carbon atoms (9, 13, 9′, 13′):



* βi = β-ionone residue.

as acetic acid (Karrer, Helfenstein, Wehrli & Wettstein, 1930). The acetic acid from the oxidation of β-carotene labelled with the [2-^{14}C]lactone was devoid of activity, indicating that the C-2 of the lactone does not equilibrate with the β-methyl carbon during incorporation into β-carotene. This agrees with observations on squalene and sterol biosynthesis (Cornforth, Cornforth, Popjak & Youhotsky-Gore, 1957; Dituri, Gurin & Rabinowitz, 1957; Amdur, Rilling & Bloch, 1957; Gould & Popjak, 1957). If C-2 of the lactone is incorporated into one *gem*-methyl group at either end of the molecule and into C-4, -4′, -8, -8′, -12, -12′, then the carboxylic acids arising from the oxidation of the β-ionone residues and the CO_2 from the residual carbons should, if one ignores the loss of some C-4 in the production of α, α-dimethylsuccinic acid, be equally labelled. In one experiment the acids and CO_2 contained 930 and 1160 c.p.m., respectively.

Isolated slices of carrot root when floated on a solution of the lactone also incorporate it into β-carotene; incorporation is again limited by failure of the slices to take up most of the substrate. The labelling pattern in the isolated β-carotene was identical with that in the β-carotene from *Phycomyes*.

We thank Dr Rita Cornforth for kindly synthesizing the labelled lactone and the Medical Research Council for financial support.

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The Proteins of the Human Adrenal Gland. By R. R. Ogilvie and R. P. Cook. (*Department of Biochemistry, University of St Andrews, Queen's College, Dundee*)

The content, nature and distribution of the proteins in human adrenal glands have been investigated. In one series of experiments apparently normal glands, removed about 3 hr. after death, were fractionated by the method of Schneider & Hogeboom (1950). The nitrogen is distributed (as approx. % of total N) as follows: supernatant fraction 35, microsomes 5, mitochondria 20, 'nuclear' layer 30. The nuclear layer consists largely of connective tissue and may be separated into 'collagen' and 'elastin' fractions. The supernatant layer contains free amino acids similar in nature to those found in human blood plasma (Dent & Walshe, 1954). In the particulate fractions the protein is apparently present as lipoprotein. On hydrolysis an amino acid pattern similar to that of the supernatant layer was obtained. The 'nuclear' layer showed large amounts of hydroxyproline. With glands stored for some time in ethanol the value for total connective tissue was (as % total N) about 69, but denaturation of the particulate fraction probably accounts for this high value. The total protein of the gland (N × 6·25) was (as % fresh wt.) 12·7 (range 9·8–14·5).

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The Biosynthesis of [^{14}C]β-Carotene. By J. GLOVER and P. P. SHAH. (*Department of Biochemistry, The University of Liverpool*)

[^{14}C]β-Carotene has been prepared previously by culturing leaves in an atmosphere containing $^{14}CO_2$ (Glover & Redfearn, 1953). Its specific activity, however, was too low for most metabolic studies. Blue-green algae, *Anabaena cylindrica* and *A. variabilis* have a higher β-carotene content (1·3–2·5 mg./g. dry wt.) which is approximately four times greater than that in plant leaves. Again, since there are only three major carotenoid pigments present, β-carotene, myxoxanthophyll and echinenone (in the proportions 55:20:25 in fresh cultures), the isolation and purification of the β-carotene from these algal lipid extracts is much easier than from that derived from leaves.

Cultures of both algae were grown in Roux bottles each containing 200 ml. inorganic medium (Fogg, 1956 and Allen & Arnon, 1955) at 28° and pH 7·0. The unicellular, *A. variabilis*, however, which requires nitrate in the medium, was the more suitable. $^{14}CO_2$/air (5 %/95 %) was circulated from a reservoir through the bottles which were continuously shaken and illuminated. Good growth (1·5–2·0 g. dry wt./l.) was obtained in 5–8 days with a 2 ml. inoculum of a growing culture. The cells were harvested and the extracted lipids chromatographed on alumina. A known amount of synthetic all-trans β-carotene (Isler, Lindlar, Montavon, Ruegg & Zeller, 1956) was added as carrier to the β-carotene fraction and the mixture crystallized to constant specific activity.

In small-scale experiments crystalline [^{14}C]β-carotene has been prepared with a specific activity (0·5 to 1·0 μc/mg. atom carbon) about one-tenth to one-half of that of the original $^{14}CO_2$ used, depending on the final dilution with carrier. Consequently, [^{14}C]β-carotene may be prepared with an activity sufficiently high for metabolic studies (Fishwick & Glover, 1957) and for the production of [^{14}C]retinene and [^{14}C]vitamin A by the methods previously used (Glover & Redfearn, 1954).

We acknowledge with thanks the gifts of the bacteria-free cultures from Dr G. E. Fogg, University College, London, and of the synthetic β-carotene from Dr O. Isler of Messrs F. Hoffman-La Roche & Co., Basel.

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A Comparison of the Serum Proteins of Normal Rats with those of Rats Bearing Liver Tumours. By P. N. CAMPBELL, BARBARA A. KERNOT and I. M. ROITT. (*Courtauld Institute of Biochemistry, Middlesex Hospital Medical School, London, W.* 1)

The composition of the serum proteins of rats bearing liver tumours induced by feeding 4-dimethylaminoazobenzene has been compared with that of normal rats by means of paper electrophoresis, zone electrophoresis and immunochemical techniques. The electrophoretic methods did not reveal any striking differences between the two types of serum. However, when an antiserum prepared by injecting rabbits with an alum precipitate of the serum from tumour-bearing rats was used to compare the composition of the serum proteins by the gel-diffusion method of Ouchterlony (1948), more lines were obtained with the tumour than with the normal serum. A simple method of demonstrating the additional lines present in the tumour serum is provided by the method of Björklund (1952, 1953, see also Feinberg, 1957) in which the lines due to the normal serum are eliminated by including some normal serum in the agar. It was found that, when the agar contained 1 part of normal serum in 100 parts of agar, the serum of the tumour-bearing rats consistently showed one major line and sometimes a faint additional line.

The component present in the serum of the tumour-bearing rats which is responsible for the line in the agar diffusion method is probably identical with the substance described by Darcy (1957) in his work on the composition of the serum proteins of tumour-bearing rats, and similar to the substance described by Nisselbaum & Bernfeld (1956) in their work with tumour-bearing mice. The sera of pregnant rats, and rats which had been subjected to partial hepatectomy when examined by the method of Björklund, also showed the additional line. Furthermore, by $(NH_4)_2SO_4$ fractionation it was possible to obtain evidence of the presence of the component in the serum from normal rats. These results are in agreement with the suggestion of Darcy that a component of the serum of the normal animal increases in concentration when there is much actively growing tissue.

In attempts to purify the component present in

the serum of the tumour-bearing rats salt fractionation was first used. The use of 18% Na_2SO_4 did not effect a substantial fractionation, but half saturation of the serum with $(NH_4)SO_4$ precipitated most of the component. The precipitated proteins could be further fractionated by zone electrophoresis on columns of treated cellulose (Flodin & Kupke, 1956; Porath, 1956) using phosphate-borate buffer pH 8·4, $I = 0·05$. Under these conditions three fractions were obtained, that with the highest mobility containing mainly albumin, and that with the lowest mainly β- and γ-globulin. The component was present in highest concentration in the middle fraction which contained mainly α_1- and α_2-globulin.

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Riboflavin Biosynthesis in *Candida flareri*. By T. W. Goodwin and D. McEvoy. (*Department of Biochemistry, The University of Liverpool*)

It has been known for some time that *Candida* spp. synthesize riboflavin in considerable amounts (Burkholder, 1943; Tanner, Vojnovich & van Lanen, 1945); *Candida flareri* N.R.R.L. 245 has now been examined in detail. Using Burkholder's medium and in shake culture, maximal growth and flavinogenesis occurs after 3 days incubation. With $(NH_4)_2SO_4$ as the sole N source, maximum growth occurs with 7·5 mg. N/100 ml. and maximum flavinogenesis with 15–20 mg. N/100 ml. Replacement of $(NH_4)_2SO_4$ by different amino acids at a level of 20 mg. N/100 ml., results in some cases (e.g. glycine, serine, aspartate, glutamate, arginine) in greatly increased growth (up to five times normal) with almost complete suppression of flavinogenesis; in other cases growth is somewhat lower than on $(NH_4)_2SO_4$ but riboflavin synthesis is again greatly reduced. When the amino acid levels are increased above 20 mg. N/100 ml., flavinogenesis is stimulated only with arginine and glycine. Urea behaves very similarly to arginine. When the growth-stimulating amino acids are added at a level of 12·5 mg. N/100 ml. to a medium containing 7·5 mg. $(NH_4)_2SO_4$–N/100 ml., flavinogenesis is lower than that on a medium containing 20 mg. $(NH_4)_2SO_4$–N/100 ml.

The importance of Fe in flavinogenesis (Tanner *et al.* 1945) has been confirmed; under our conditions Fe levels of 0·1 p.p.m. are strongly inhibitory. The reduced riboflavin production previously reported on media containing high glucose and phosphate levels (Burkholder, 1943) appears to be due to the increased Fe content of the media.

In the presence of $(NH_4)_2SO_4$, xanthine is strongly flavinogenic, hypoxanthine and uric acid are as effective as $(NH_4)_2SO_4$, while adenine and adenosine are ineffective. In the absence of $(NH_4)_2SO_4$, adenine and hypoxanthine do not support growth, while xanthine and uric acid support both growth and flavinogenesis. Pyrimidines do not support growth and are non-flavinogenic in the presence of $(NH_4)_2SO_4$.

These results may be compared with those obtained in similar investigations on *Eremothecium ashbyii* (Goodwin & Pendlington, 1954; Brown, Goodwin & Pendlington, 1955).

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The Metabolic Stability of Nucleic Acids in Cells Grown in Tissue Culture. By R. Y. Thomson and John Paul. (*Department of Biochemistry, The University of Glasgow*)

In spite of intensive study it is still uncertain whether deoxyribonucleic acid (DNA), once synthesized, is metabolically stable (Siminovitch & Graham, 1956). In an attempt to answer this question Earle's 'L' strain cells (Sanford, Earle & Likely, 1948) were grown in the presence of [^{14}C]formate. The cells were then allowed to grow for eight generations in non-radioactive medium, samples being taken for

analysis at intervals. Since in rapidly growing cultures any breakdown products are likely to be reincorporated into newly synthesized materials, the cells were grown rather slowly (mean generation time: 4–7 days). During this period of growth in non-radioactive medium the total amount of isotope in the DNA adenine, guanine and thymine of the cultures declined slowly so that after a 256-fold increase in cell number these bases still retained 40–80% of their original activity. On the other hand, the total amount of isotope in the ribonucleic acid (RNA) adenine and guanine fell sharply throughout the experiment. These results appear to indicate that DNA once synthesized is metabolically relatively stable whereas at least a part of the RNA is continuously being broken down and replaced by the products of new synthesis.

Even in such experiments with slowly growing cultures it is possible that if there is any breakdown of DNA the labelled breakdown products, e.g. deoxynucleosides and deoxynucleotides, might be immediately re-utilized in the synthesis of new DNA, so that there would be no overall loss of ^{14}C from the DNA of the culture. In order to meet this difficulty similar experiments were carried out in which the cells, after being labelled, were grown for nine generations in non-radioactive medium in presence and absence of non-radioactive thymidine. Under these circumstances it was found that the DNA thymine retained about 75% of its initial activity in cells grown in absence of thymidine, but only about 50% in cells grown in presence of thymidine. It would appear, therefore, that in these experiments there was a slow breakdown of DNA (about 10% per generation) which, in the absence of thymidine, might be masked because of the re-utilization of breakdown products for synthesis of new DNA.

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The Incorporation of [^{14}C]Formate into the Nucleic Acids of the Mouse Ehrlich Ascites Carcinoma *in vivo* and *in vitro*. By R. Y. Thomson, R. M. S. Smellie and Helen Harrington. (*Department of Biochemistry, The University of Glasgow*)

It has previously been reported that *in vivo*, the Ehrlich ascites carcinoma incorporates [^{14}C]formate rapidly into the purines of deoxyribonucleic acid (DNA), nuclear ribonucleic acid (nRNA) and cytoplasmic ribonucleic acid (cRNA) as well as into the DNA thymine, whereas *in vitro*, incorporation of [^{14}C]formate into the purines of DNA, nRNA and cRNA is greatly reduced (Smellie, Thomson, Goutier & Davidson, 1956). Experiments have been carried out to determine the distribution of ^{14}C in the adenine from the tumour nucleic acids after the incorporation of [^{14}C]formate *in vivo* and *in vitro*. *In vivo* it was observed that the isotope was approximately evenly distributed between carbon atoms 2 and 8 of the purine ring, whereas *in vitro* the ratio of the activity in carbon 2 to that in carbon 8 was in the region of 4:1, indicating that *in vitro* incorporation of formate represented primarily the terminal stages of purine ring biosynthesis.

In accord with these findings it was observed that the addition of 4-amino-5-iminazole carboxamide or its riboside to suspensions of the tumour cells *in vitro* greatly stimulated formate incorporation into the nucleic acid and acid-soluble purines, whereas the addition of glycinamide ribotide or riboside (earlier intermediates in the pathway of purine biosynthesis, Hartman, Levenberg & Buchanan, 1956) was without effect on formate incorporation into the nucleic acid purines. This suggests that some step in the conversion of glycinamide ribotide to the carboxamide ribotide is rate-limiting in the ascites cells *in vitro*.

It has also been found that the addition of glucose in concentrations of the order of 5 μmole/ml. to the tumour suspensions *in vitro* stimulates uptake of formate, adenine and glycine into the tumour nucleic acids although it is without effect on the incorporation of inorganic ^{32}P.

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Amylases of Holotrich Protozoa from Sheep Rumen. By D. L. MOULD and GWEN J. THOMAS. (*Rowett Research Institute, Bucksburn, Aberdeenshire*)

Zone electrophoresis of aqueous extracts of freeze-dried holotrich protozoa (Heald & Oxford, 1953) has been carried out on starch columns according to the method of Flodin & Porath (1954). In sodium phosphate buffer pH 7·0, $I = 0{\cdot}05$, two well-defined zones of α-amylase activity with differing mobility were observed. It has been shown that each of these α-amylases is specific to the *Isotricha* and *Dasytricha* present in the mixed preparation. The optimum pH of action of isotrich amylase was 4·8 while dasytrich amylase has two optima at 5·0 and 6·0. The activity is independent of the presence of Cl^- or Ca^{2+} ions.

The isoelectric point for isotrich amylase was estimated as 5·4. Both these amylases may be grouped with the amylases of *Aspergillus oryzae* and the rumen streptococcus in the broad classification of Fischer & de Montmollin (1951).

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Biochemical Studies of Individual Genera of Rumen Holotrich Protozoa. By B. H. HOWARD. (*Rowett Research Institute, Bucksburn, Aberdeenshire*)

Eadie & Oxford (1957) have described how a sheep was treated so that its rumen contained only one holotrich species, *Dasytricha ruminantium*, instead of the usual mixed population of *D. ruminantium*, *Isotricha intestinalis* and *I. prostoma*. Another sheep was similarly treated to contain only the isotrichs. Suspensions containing only one genus of protozoa were obtained from these sheep by the method of Heald, Oxford & Sugden (1952).

In manometric experiments (Heald & Oxford, 1953), the two genera differed in their actions on galactose, cellobiose and maltose. The rates at which these sugars were fermented by *Dasytricha ruminantium* were 80, 100 and 33 %, respectively of the rate of fermentation of glucose. The isotrichs did not ferment any of these sugars. Galactose is the only carbohydrate fermented by rumen holotrichs which is not a derivative of glucose or fructose. The fermentation products resemble those from glucose, and include storage starch, lactic acid, volatile acids (almost wholly acetic) and CO_2. The fermentation rates of the β-glucosides aesculin, amygdalin, arbutin and salicin by *D. ruminantium* were respectively, 50, 90, 60 and 90 % of the glucose fermentation rate; the corresponding rates of fermentation by the isotrichs were 4, 13, 30 and 25 %. Isotrichs fermented rice starch grains readily without lag, but holotrich starch grains only slowly. *D. ruminantium* did not ferment either kind of starch. These results confirm conclusions drawn from microscopic observation of the behaviour of holotrichs towards starch grains (Sugden & Oxford, 1952). Mannose and glucosamine, after a short lag, partly suppressed the endogenous fermentation of both genera. Sugden & Oxford (1952) found these sugars to be toxic to holotrichs.

Freezing and thawing burst the protozoa, yielding a cell-free extract. The *D. ruminantium* extract, but not the isotrich extract, contained cellobiase and maltase. β-glucosidase activity was present in both extracts; activity/mg. protein N was approx. ten times as great in the *D. ruminantium* extract as in the isotrich extract, whether the substrate was *o*-nitrophenyl-β-glucoside (Conchie, 1954), aesculin, arbutin or salicin. Glucose was produced from amygdalin by the *D. ruminantium* extract.

Both genera produced an invertase. The isotrichs were particularly active, some extracts hydrolyzed over 4 g. sucrose/mg. protein N in 30 min. at 39°, pH 5·5. Four fructose-containing substances, of at present undetermined constitution, were synthesized during the action of each of the extracts on a 20 % sucrose solution. Both extracts also hydrolyzed laminaribiose, raffinose, inulin, bacterial levan and starch, but not melibiose, lactose, trehalose or xylobiose.

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An Invertase from the Holotrich Protozoa of Sheep Rumen Liquor. By A. O. CHRISTIE and J. W. PORTEOUS. (*Department of Biological Chemistry, University of Aberdeen*)

Essentially bacteria-free holotrich protozoa (*Isotricha* and *Dasytricha* spp.) were isolated by the technique of Heald, Oxford & Sugden (1952) from rumen-fistulated hay-fed sheep. A cell-free invertase obtained from the protozoa showed an optimal pH of 5·2 in 0·05M-acetate buffer at 37° for hydrolytic activity on 0·025M-sucrose; these conditions were used throughout the present experiments except for variation of the sucrose concentration between 0·002 and 1·0M. The enzyme preparation contributed not more than 2 μg. protein-nitrogen/ml. assay system.

Hydrolase activity was determined directly on samples from the assay solutions by the colorimetric technique of Nelson (1944). Chromatographic analyses of the assay solutions for oligosaccharides, sucrose, glucose and fructose were performed by slight modifications of the methods of Bealing & Bacon (1953).

Hydrolase activity of the protozoal invertase was maximal at 0·025M-sucrose then declined until at 0·05M-sucrose it was approximately 70% of the maximal value and remained at this level between 0·05 and 1·0M-sucrose.

Transferase activity in the protozoal enzyme preparation, as evidenced by the appearance of a single oligosaccharide spot on chromatograms, was not apparent at initial sucrose concentrations below 0·1M and then only after at least 16% apparent hydrolysis of the sucrose had taken place. Preliminary experiments suggested that oligosaccharide formation was optimal at 0·25M-sucrose when four oligosaccharides appeared under conditions such that approximately 50% apparent hydrolysis of sucrose had occurred; together these oligosaccharides probably accounted for not more than 5% of all sugars present.

Experiments with ethanol (10% v/v) as additional substrate to 0·25M-sucrose showed the presence of a non-reducing 'fast-running spot' on chromatograms, probably ethyl-β-D-fructoside (cf. Bacon, 1952; Bealing, 1953). Analysis of this material showed that its formation accounted for only 2% inhibition of hydrolase activity by 10% (v/v) ethanol; the observed inhibition of hydrolase activity was 44%.

These results on the hydrolase and transferase activities of the protozoal enzyme contrast markedly with those obtained in parallel experiments with a yeast invertase; these latter results accorded with those originally reported by Bacon & Edelman (1950) and by Blanchard & Albon (1950), and since elaborated by many workers (see Edelman, 1956, for a review).

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A Lipophosphoprotein Complex in the Plasma of the Domestic Fowl. By W. M. MCINDOE. (*A.R.C. Poultry Research Centre, King's Buildings, Edinburgh* 9)

A lipophosphoprotein complex can be readily precipitated from the plasma of laying hens by dilution with 5–10 vol. water. A similar observation has been made by McKinley, Oliver, Maw & Common (1953) in oestrogenized pullets and by Schjeide & Urist (1956) in oestrone-treated cocks. The material is not present in non-laying birds. The complex can be dissolved in water and reprecipitated in 0·09% NaCl.

Preliminary analyses of samples from hens in full production showed the complex to have a ratio mg. N/mg. P consistently close to 3·5.

The material is present to the extent of about 2 g.% and contains about 20% protein (N, 14·5%; P, 0·65%). The lipid component has the approximate composition: 25% phospholipid, 4% cholesterol, the remainder being presumably neutral fat.

The lipoprotein accounts for 80% of plasma total lipid and must represent almost all the lipid in excess of that in the plasma of the non-laying bird. About 10% of the protein and 30% of the phosphoprotein phosphorus of the plasma are present in the complex.

During the period when fowls are going off lay the amount of precipitable lipoprotein decreases and markedly so about the time the last egg is laid. Subsequent to this the material precipitated consists almost entirely of protein (containing no phosphorus) and this in turn disappears.

Precipitable lipoprotein can be detected in plasma a week before the first ovulation and the concentration during the immediately preovulatory period is higher than when egg-laying is established.

The appearance of the lipoprotein just prior to the commencement of egg-laying, its disappearance in non-laying birds and its composition all point to its being a constituent of egg yolk. Some of its properties are shared with 'lipovitellin'.

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Observations on the 'Non-protein Nitrogen' of Fowl's Blood. By D. J. Bell (introduced by W. M. McIndoe). (*A.R.C. Poultry Research Centre, King's Buildings, Edinburgh* 9)

Some details of two general observations will be reported. First, the values obtained for the NPN of whole blood depends on the method used for deproteinization, in agreement with the findings of Hunter (1957) on human blood. Secondly, since the erythrocytes carry about 75% of the whole-blood NPN, the latter is markedly affected by alterations in the relative volumes of plasma and red cells (Bell, 1957) without concomitant changes in the plasma NPN. Measurements of pathological changes of NPN in fowl blood should therefore take into account the volume percentage of erythrocytes. Virtually identical NPN values are found in trichloracetic acid blood filtrates and in dialysates (cellophan) of filtrates made by Hunter's (1956) method.

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The Effect of Feeding Tung Oil on the Composition of the Lipids of the Milk and Plasma of Cows. By G. A. Garton, W. R. H. Duncan and A. K. Lough. (*Rowett Research Institute, Bucksburn. Aberdeenshire*)

Three lactating Ayrshire cows, fed on silage and turnips, were each given 100 g. tung oil (containing 79·8% α-elaeostearic acid, i.e. octadeca-9, 11, 13-trienoic acid) as a drench daily for 6 days. Milk samples were obtained daily from 4 days before to 14 days after tung oil administration. Blood samples were taken immediately before the first drench of tung oil and 17 hr. after the last drench. Plasma lipids were separated into acetone-soluble and acetone-insoluble (phospholipid) fractions and the former chromatographed on silicic acid to yield sterol esters and triglycerides (cf. Lough, Garton & Duncan, 1957). The lipids of the milk and the fatty acids of the plasma lipid fractions were examined spectrophotometrically for conjugated di- and tri-enoic acids, using the extinction values given by Hilditch (1956).

Milk fat usually contains traces of conjugated di- and tri-enoic fatty acids and in these experiments the values were about 0·7% and about 0·06%, respectively, before tung oil was administered. In agreement with the findings of Houston, Cotton, Kon & Moore (1939), the conjugated trienoic fatty acid content of the milk fat increased when tung oil was administered, reaching maximum values of 0·18, 0·21 and 0·33%, respectively, in the three animals. However, in contradistinction to the findings of Houston *et al.* (1939) that no increase in the conjugated dienoic content of the milk fat could be detected, we observed an increase in the content of this species of acid to values of 1·6, 1·7 and 2·4%. The maximum values for both conjugated dienoic and trienoic acids were reached at about the time of the last drench of tung oil and were followed by a gradual return to normal values. The values for conjugated dienoic acid declined less rapidly than did those for conjugated trienoic acid, suggesting that partially hydrogenated elaeostearic acid (probably formed in the rumen, cf. Shorland, Weeninck & Johns, 1955) was being gradually absorbed, though it is possible that partial reduction of elaeostearic acid took place in the depots or elsewhere, as has been reported in rats (Miller & Burr, 1937) and in hens (Cruickshank, Houston & Moore, 1939; Reiser, 1951).

Following tung oil feeding, increased amounts of conjugated dienoic acid were found in all three plasma lipid fractions; increased amounts of conjugated trienoic acid were found in the sterol esters, possibly in the phospholipids, but not in the glycerides.

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The Preparation of Proteins and Peptides containing S-Sulphonate Groups.

By J. LEGGETT BAILEY. (*The Rockefeller Institute for Medical Research, New York, and The Rowett Research Institute, Bucksburn, Aberdeenshire**)

Structural studies (Sanger, 1949; Hirs, Stein & Moore, 1956; Kimmel, Thompson & Smith, 1955) on proteins containing cystine have been greatly facilitated by preliminary treatment of the protein with performic acid, whereby all the half-cystine residues are converted to cysteic acid residues. Reductive rather than oxidative splitting of S—S bonds may be preferable when the protein contains tryptophan. The following is a preliminary report on a study of the reaction of sulphites with the S—S groups of proteins and peptides. The ultimate aim of the work is to introduce S-sulphonate ($S \cdot SO_3H$) groups into proteins by the following cycle of operations

$$RSSR + SO_3^{2-} \longrightarrow RS^- + RSSO_3^-$$
$$\uparrow \quad S_4O_6^{2-} \quad \downarrow$$

or o-$C_6H_4(CO_2H)IO$

Experiments with simple compounds such as cystine and oxidized glutathione indicate that a near quantitative conversion to the corresponding S-sulphonates can be achieved by reaction with 30–40 mol. excess of both the sulphite and the oxidizing agent at pH 7–8. The extent of reaction can be followed by ion-exchange chromatography using the resin Dowex 1-X8 and 0·1M pH 3·1 sodium monochloroacetate as eluent.

The disulphide groups of insulin are cleaved by excess sodium sulphite and sodium tetrathionate in 8M-urea in the presence of cysteine at pH 7·6. The *A* and *B* chains containing $S \cdot SO_3H$ groups can be separated in 80–85% yield by passage through a 3·0 × 1·0 cm. column of Dowex 50-X2 equilibrated in 8M-urea buffered at pH 3·0 with 0·2M-citrate. The *A* chain passes through and the *B* chain is eluted with 8M-urea buffered at pH 7·6.

Analytical techniques basic to this work are amperometric titration of SH groups with silver nitrate (Benesch, Lardy & Benesch, 1955) and direct estimation of the S-sulphonate group. The latter involves electrodialysis of the protein derivative to remove the free sulphur-containing anions, oxidation of the S-sulphonate groups with performic acid and the determination of the liberated sulphate by a gravimetric procedure. Under conditions that give good yields of the insulin chains, a product has been obtained from bovine serum albumin that yields sulphate corresponding to 32 S-sulphonate equivalents per 65 000 g.

* Present address.

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The *N*-Acetyl-β-galactosaminidase Activity of Two Sources of *N*-Acetyl-β-glucosaminidase.

By R. HEYWORTH, J. BOROOAH and D. H. LEABACK. (*Department of Biochemistry, The Institute of Orthopaedics, Royal National Orthopaedic Hospital, Stanmore, Middlesex*)

p-Nitrophenyl *N*-acetyl-β-galactosaminide has been prepared from galactosamine hydrochloride through 1-chloro-tetra-acetyl galactosamine by a method similar to that described by Leaback & Walker (1957) for the corresponding glucosaminide. The liberation of *p*-nitrophenol on incubation of these compounds with tissue preparations in 0·05M citrate buffer at 37° has been used to estimate their galactosaminidase and glucosaminidase activities.

Both activities were present in extracts of ram testis and sweet almond meal (emulsin). In the case of the testis extract the pH optima for the hydrolysis of galactosaminide and glucosaminide were 4·3 and 4·4 and the K_m values were 0·36 mM and 1·48 mM, respectively. The corresponding values for emulsin were 4·3 and 4·4, 0·44 mM and 0·45 mM.

Using equimolar substrate concentrations the ratio of galactosaminidase to glucosaminidase activity of the testis extract was 1:4. This ratio remained constant in four fractions prepared by ammonium sulphate precipitation, and also on progressive inactivation to 30% of the original activity by pre-incubation in citrate buffer.

With both enzyme preparations substrate com-

petition was demonstrated, the *p*-nitrophenol liberated in the presence of a mixture of galactosaminide and glucosaminide being intermediate between the amounts liberated from each substrate individually. Phenyl *N*-acetyl-β-glucosaminide inhibited the hydrolysis of each *p*-nitrophenyl substrate.

Both galactosaminidase and glucosaminidase activities of the enzyme preparations were inhibited by *N*-acetylgalactosamine and by *N*-acetylglucosamine, the former being the more powerful inhibitor. In the case of the testis extract these end-products had less inhibitory effect on the hydrolysis of galactosaminide than on the hydrolysis of glucosaminide. A powerful inhibitor of glucosaminidase, 2-acetamido-2-deoxygluconolactone (Marsh & Levvy, 1957), at a concentration of 0·02 mM caused inhibitions of 23 and 64% respectively in the hydrolysis by testis extract of 2·2 mM galactosaminide and glucosaminide.

It seems probable that, in these preparations, one enzyme has both galactosaminidase and glucosaminidase activities.

We are indebted to Dr G. A. Levvy for a gift of 2-acetamido-2-deoxygluconolactone and to Messrs Bengers Ltd., for generous supplies of freeze-dried testis extract.

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Factors Influencing the Paper-Chromatography of Amino-Sugars. By D. H. Leaback and P. G. Walker. (*Department of Biochemistry, The Institute of Orthopaedics, Royal National Orthopaedic Hospital, Stanmore, Middlesex*)

Waldron-Edward (1954) showed that in the presence of sulphate, glucosamine gave two amino-sugar spots on chromatograms developed with weakly basic or acidic solvents. In the present work, ionic interactions between amino-sugar salts and chromatographic solvents have been investigated.

The behaviour of glucosamine-free base, hydrochloride, sulphate and acetate applied individually to paper chromatograms was studied by localizing amino-sugar, chloride and sulphate after developing with various solvents.

In phenol-ammonia (Partridge, 1948) glucosamine ran as the free base irrespective of the form in which it was applied to paper (R_F values: amino-sugar, 0·66; chloride, 0·19; sulphate, 0·08). In a strongly acid solvent (butanol–ethanol–1 N-HCl: 40:11:19) each compound ran as glucosamine hydrochloride (R_F values: amino-sugar and chloride, 0·18; sulphate, 0·52).

In weakly basic or acidic solvents amino-sugar salts moved in some cases as a single spot in the form of the ion-pair applied to the paper, while in other cases double spots were produced as a result of interactions with the solvent. In butanol–pyridine–water: 6:4:3, the R_F of glucosamine-free base was 0·32. The hydrochloride gave a single spot (R_F, 0·29) containing both amino-sugar and chloride. Glucosamine sulphate gave two amino-sugar spots (R_F, 0·31 and 0·09) and sulphate trailed from R_F, 0·09 back to the origin, indicating the formation of some glucosamine-free base and pyridinium sulphate. Whereas in butanol–acetic–water: 4:1:5, glucosamine-free base and glucosamine acetate gave single spots, both glucosamine hydrochloride and sulphate gave double amino-sugar spots due to the formation of some glucosamine acetate by interaction with the solvent (cf. Pollard, McOmie, Martin & Hardy, 1955). The behaviour of glucosamine and galactosamine hydrochlorides was qualitatively similar in every respect.

Caution is therefore required in the interpretation of unusual amino-sugar spots on chromatograms developed with weakly basic or acidic solvents, especially if the nature of the anions present is uncertain.

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The Formation of δ-Aminolaevulic Acid by Chicken Erythrocytes *in vitro*. By W. G. Laver and A. Neuberger. (*Department of Chemical Pathology, St Mary's Hospital Medical School, London, W. 2*)

Experiments carried out by Shemin, Rimington, Falk and their co-workers and by our own group, have indicated strongly that in the biosynthesis of porphyrins glycine is condensed with succinate to give α-amino-β-oxoadipate (AOA), the latter being decarboxylated to δ-aminolaevulic acid (ALA) which is then converted through porphobilinogen to porphyrins. Experiments to be reported elsewhere have shown that AOA has a very short chemical half-life and in the present work we have concentrated on demonstrating the formation of ALA.

Erythrocytes from chickens which had been made anaemic by injection of phenylhydrazine were washed, lysed and homogenized. On centrifuging

at 3500 *g* three layers were obtained: an almost clear supernatant (*S*), a top layer of particles (P_1) and a bottom layer of particles (P_2). The fractions were incubated in air with [α-^{14}C]glycine, succinate, $MgCl_2$, phosphate and an excess of unlabelled ALA. The ALA was then converted into 2-methyl-3-carbethoxy-pyrrol-4-(β-propionic acid) (Mauzerall & Granick, 1956) and its radioactivity determined. Well-washed P_1 showed low activity which was increased about 3·5 times by addition of *S* which was inactive on its own, and to a lesser extent (increase of about 50%) by addition of a boiled extract or ultrafiltrate of *S*; P_2 showed low activity under all these conditions. The activity of P_1 alone could also be increased 2·5 times by addition of pyridoxal phosphate and coenzyme A. Net formation of ALA by washed P_1 without the use of carrier ALA and labelled glycine was also shown by a modification of the colorimetric method of Shuster (1956) in experiments carried out in this laboratory by S. Udenfriend and one of us (A.N.) The nature of the active particles in fraction P_1 and the detailed mechanism of this condensation are being investigated.

One of us (W.G.L.) wishes to thank the Australian Government for a C.S.I.R.O. studentship.

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Some Properties of the Malaria Parasite of Rodents, Free of its Host Cell the Reticulocyte.
By I. B. R. Bowman, Linda R. Cook, P. T. Grant and W. O. Kermack. (*Department of Biological Chemistry, University of Aberdeen*)

Little is known about the metabolism of *Plasmodium berghei* and *in vitro* observations on intact infected reticulocytes are not easy to interpret because the presence of the parasite may influence the metabolic activity of the host cell.

In the present experiments, the parasites were obtained apparently free of blood cells by haemolysis of a washed suspension of infected cells with the serum of rabbits previously immunized against mouse or rat erythrocytes. Fresh guinea-pig serum was added as a source of complement. The free parasites were isolated by centrifugation and were then washed in saline containing glucose. These parasite preparations were essentially free of leucocytes and unlysed red cells as judged by microscopic examination of stained smears. Some parasites, however, still appeared to be attached to the membrane or stroma of the original host cell. Although not infective when injected intraperitoneally, a heavy parasitaemia invariably developed in the mouse when a free parasite suspension was administered intravenously. These observations indicated that unlysed infected cells are absent and that a proportion of the free parasites were capable of invading fresh reticulocytes.

These parasite preparations were metabolically active. For example, when incubated at 37° in saline containing 0·2% w/v glucose, the molar ratio of glucose utilized to lactate produced was in the range 1·0–1·4 in five individual experiments. This is a similar ratio to that found previously by Fulton & Spooner (1956) for intact infected reticulocytes. Preliminary studies also indicated the presence of intracellular proteolytic enzymes in these preparations. Using the method of Anson (1938), it was found that water extracts of fresh or freeze-dried parasite material hydrolyzed urea-denatured haemoglobin. In contrast, similar extracts of the stroma of red cells of non-infected rats with an induced reticulocytosis (Sherwood-Jones, Maegraith & Gibson, 1953) were inactive. The proteolytic activity in extracts of the parasite had a minor pH optimum at pH 3·8 and a major optimum at pH 7·6. At this latter pH value, the comparable activity of several urea-denatured protein substrates was as follows: reticulocyte haemoglobin, 64, and globin, 100. Mature erythrocyte haemoglobin, 20, and globin, 80. The proteolytic activity of these extracts towards native haemoglobins or globins was barely measurable.

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Chromatography of Steroid Conjugates. By I. E. Bush. (*Radcliffe Infirmary, Oxford*)

Most of the important steroid metabolites in urine are conjugated and are usually estimated after hydrolysis to free steroids. It would be a great advantage if the conjugates themselves could be extracted and separated for estimation. Some steroid conjugates have been separated on paper chromatograms by Lewbart & Schneider (1955), but their solvent systems had little resolving power. Kellie & Wade (1956) separated some 17-oxosteroid glucuronosides on alumina after methylation and acetylation, these preliminaries being a major disadvantage.

Encouraged by Martin's (1949) theoretical treatment attempts were made to separate steroid conjugates without any pretreatment. It has been found that conjugates are but slightly more difficult to separate than the free steroids; furthermore, the relative effects of features of the steroid moiety are the same in the conjugates as in the free steroids. Using pure conjugates, and conjugates isolated from human urine, solvent systems were devised capable of separating all the major 17-oxosteroid glucuronosides and sulphates, the glucuronosides of tetrahydrocortisone and tetrahydrocortisol, and other unidentified conjugates. Apart from the expected glucuronosides and sulphates there seem to be at least two other classes of conjugate in human urine.

Of the numerous solvent systems tried out over the last four years the following were most useful:

(1) 2N aqueous ammonium hydroxide and an equal volume of *n*-butanol, or methyl ethyl ketone, or ethylene dichloride : *tert.*-butanol (3:1, v/v).

(2) Toluene:*tert.*-butanol:acetic acid:water in mixtures ranging from 75:25:30:70 to 85:15:30:70 (by volume).

(3) Ethylene dichloride : *tert.*-butanol : acetic acid : water in mixtures ranging from 75:25:30:70 to 90:10:30:70 (by volume).

In all cases the less polar phase was the mobile phase. The systems were run for 3–24 hr. at 18–26° on Whatman papers 2, 3, 31, and 3M, after 3–15 hr. equilibration. They have also been used on celite columns for preparative work. Closely related conjugates were best separated in acid systems giving R_F values of about 0·25. Group separations were conveniently made with alkaline systems, in which the sulphates move with high R_F values permitting their complete separation from the glucuronosides. Slight tailing of sulphates in acid systems was rarely serious but was conveniently stopped by adding HCl to the system (1% of total volume).

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The *in vitro* Effect of Di-(2-chloroethyl)methylamine (HN2) on Protein and Ribonucleic Acid Metabolism in Rat Liver. By G. A. J. Goodlad and H. N. Munro. (*Department of Biochemistry, The University, Glasgow, W.* 2)

Nitrogen mustards have been shown to inhibit various biosynthetic mechanisms in rat-liver slices when present at concentrations at which they have little effect on respiration (McKinney, 1949). Crathorn & Hunter (1956), on the other hand, found that nitrogen mustards did not inhibit the incorporation of amino acids into protein by *Staph. aureus* under conditions of net protein synthesis although incorporation by 'exchange reactions' was inhibited.

In the present work the effect of HN2 on the uptake of [^{14}C]glycine into protein was studied in rat-liver slices incubated for periods of up to 3 hr. The uptake of ^{32}P into ribonucleic acid phosphorus (RNAP) was studied in parallel. Although uptake of isotopes into protein and RNAP was inhibited by 10^{-3}M HN2 complete inhibition could not be obtained. The incorporation-time curves suggested that the inhibitory action occurred more slowly than the initial uptake of isotope. HN2 inhibited the incorporation of glycine into protein at concentrations above $6{\cdot}25 \times 10^{-5}$M, while the incorporation of ^{32}P into RNAP appeared more sensitive. Comparison of our data with those of McKinney (1949) suggests that the incorporation of glycine into protein is more sensitive to HN2 than is the conjugation of glycine with *p*-aminobenzoic acid to form *p*-aminohippuric acid.

The fact that so many synthetic systems are affected by HN2 led us to consider whether the inhibitory action is a general one affecting energy donors. We therefore examined the effect of HN2 on the incorporation of glycine into liver protein using the homogenate system of Zamecnik & Keller (1954), in which energy is supplied by 3-phosphoglyceric acid and adenosine triphosphate. Under these conditions even 10^{-3}M HN2 failed to exert any significant inhibitory effect.

In addition, the possibility of differences in sensitivity between cytoplasm and nucleus was investigated since it is well known that mustards cause mitotic inhibition in the whole animal at dose levels having no apparent effect on the rest of the cell. A study of the effect of different concentrations of HN2 on the uptake of [^{14}C]glycine into the protein of nuclei and cytoplasm derived from liver slices after incubation, however, failed to show any apparent difference in sensitivity.

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PROCEEDINGS OF THE BIOCHEMICAL SOCIETY

The **366th Meeting** *of the Biochemical Society was held in the Physiology Lecture Theatre, Guy's Hospital Medical School, London, S.E.* 1, *on Saturday,* 19 *October* 1957, *starting at* 2 *p.m., when the following papers were read:*

COMMUNICATIONS

The Isolation of Androst-5-ene-3β:16β:17β-triol from the Urine of Normal Men. By K. Fotherby. (*Department of Biochemistry, University of Edinburgh*)

Fotherby, Colas, Atherden & Marrian (1957) have proposed that 16α-hydroxydehydro*epi*androsterone is an intermediate in the metabolic formation of androst-5-ene-3β:16α:17β-triol from dehydro*epi*-androsterone. This transformation is analogous to that suggested by Marrian, Watson & Panattoni (1957) and Marrian, Loke, Watson & Panattoni (1957) for the formation of oestriol from oestrone in which 16α-hydroxyoestrone is the intermediate. Furthermore Marrian, Watson & Panattoni (1957) have suggested that 16β-hydroxyoestrone might be the intermediate in the formation of 16-*epi*-oestriol (Marrian & Bauld, 1955) from oestrone. It seemed possible, therefore, that androst-5-ene-3β:16β:17β-triol might arise metabolically from dehydro*epi*androsterone via 16β-hydroxydehydro-*epi*androsterone and accordingly androst-5-ene-3β:16β:17β-triol was searched for in the urine of normal men.

The residue from a chloroform extract of a large volume of urine which had been boiled (without adjustment of the pH) for 2 hr. was subjected to a Girard separation to remove the ketonic fraction. By countercurrent distribution of the non-ketonic fraction in two different solvent systems a fraction was obtained which was slightly less 'polar' than androst-5-ene-3β:16α:17β-triol and gave a positive Pettenkofer test. This fraction after chromatography on a magnesium silicate-Celite column yielded a crystalline substance which had m.p. 259–262° (uncorr.) unchanged by admixture with authentic androst-5-ene-3β:16β:17β-triol. It formed an acetate, m.p. 206–209° (uncorr.), $[\alpha]_D^{18}$ −35° in ethanol (*c*, 0·20). (Found: C, 69·3; H, 8·3. Calc. for $C_{25}H_{36}O_6$: C, 69·4; H, 8·3 %). The mixed m.p. with authentic androst-5-ene-3β:16β:17β-triol triacetate (m.p. 208–211°) was 206–209° (uncorr.) and the spectra of solutions of the acetates in conc. H_2SO_4 were identical.

Androst-5-ene-3β:16β:17β-triol is the first neutral steroid with a hydroxyl group in the β configuration at position 16 to be isolated from the urine of human beings. Based upon the amount of crystalline material obtained its concentration in the urine of normal men is about 5 μg. per litre.

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Composition of Cardiolipin. By M. G. Macfarlane and G. M. Gray. (*Lister Institute of Preventive Medicine, London*)

The complex phosphatidic acid, cardiolipin, was considered by Pangborn (1947) to be a glyceryl-glycerophosphate derivative containing three phosphoric acid molecules linking four glycerol molecules, the latter being also esterified with five linoleic and one oleic acid residues (structure I, represented as: $G(R_1R_2)$–P–$G(R_3)$–P–$G(R_4)$–P–G (R_5R_6)). We have obtained cardiolipin (P, 4·1 %; N < 0·05 %; inositol, nil) from ox-heart muscle by chromatography on silicic acid, and by the methods of Pangborn (1945) and Faure & Morelec-Conlon (1956), and find a molar ratio P:glycerol:fatty acids of 1:1·5:2. This suggested that the molecule contains two phosphoric acid molecules linking three glycerol residues, four of the remaining five alcoholic groups being esterified with fatty acids (e.g. structure II: $G(R_1R_2)$–P–G(OH)–P–$G(R_3R_4)$). As analytical values for P, etc., are inconclusive in establishing either structure, other evidence has been sought.

(1) On saponification in N-NaOH, followed by N-HCl 15 min. at 100°, cardiolipin is split into fatty acids, glycerophosphate and glycerol (Found: free glycerol, 30 % of total; inorganic P, nil; 98 % P as monophosphoric ester). Structure I requires 25 %, II 33 % free glycerol.

(2) Structure II requires an alcoholic group, susceptible to oxidation. Cardiolipin was hydrogenated and treated with $KMnO_4$ in 0·1 M-acetate buffer, pH 5·8, 16 hr. at 38°. The product formed a complex with the MnO_2, and only 70 % of the P was recovered, contaminated with Mn; ratio fatty acid ester/P, 2:1. After hydrolysis of this material in 2 N-HCl, 2 hr. at 100°, the solution gave a positive test for methyl glyoxal (Ariyama, 1928) indicating the presence of a dihydroxy-acetone residue in the oxidation product, but only 12 % of the calculated amount was found. Methyl glyoxal, however, is destroyed at 100° in N-HCl containing traces of MnO_2.

The results are therefore at present inconclusive, but throw doubt on Pangborn's structure (I).

Examination of the mixed fatty acids of cardiolipin (75·4 % by wt., iodine no. 165) by alkali isomerization, and by gas-liquid chromatography of the methyl esters, indicated the constituents as: (%) palmitic 0·5; stearic 0·8; palmitoleic 5·2; oleic 11·0; linoleic 72·0; linolenic 8·0; C_{20} polyenoic, 1·5. The results suggest that cardiolipin is not a single compound, but a mixture of similar compounds varying in fatty acids.

We are indebted to Dr A. T. James and Dr J. Webb, National Institute for Medical Research, for the chromatography of the fatty acids. Acknowledgement is made to the Medical Research Council for a grant to M.G.M. for the assistance of G.M.G.

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The Position of the Aldehyde Residue in Natural Plasmalogens. By G. M. Gray (introduced by M. G. Macfarlane). (*Lister Institute of Preventive Medicine, London*)

Klenk & Debuch (1954, 1955) and Debuch (1956) showed conclusively that in natural plasmalogens (acetalphospholipids) a fatty acid as well as a fatty aldehyde residue is linked to the glycerophosphorylcholine (or ethanolamine) moiety. The action of phospholipase A on plasmalogen-rich lecithin (Rapport & Franzl, 1955, 1957) indicated an α-acyl-β-aldehyde structure. Proof of this structure has now been obtained.

The choline-containing phosphatide fraction (53 % lecithin, 47 % choline-plasmalogen), obtained by chromatographic fractionation of ox-heart phospholipid on silicic acid, was treated with 90 % (v/v) acetic acid at 37° for 17 hr. The lysolecithin, derived thus solely from choline-plasmalogen by liberation of aldehyde, was separated quantitatively from the intact lecithin and the free aldehyde on a silicic acid column (solvent $CHCl_3$–MeOH, 1:1, v/v), and hydrogenated. The product (12·0 mg. P) was oxidized with $KMnO_4$ in 0·1 M-acetate buffer, pH 5·8, for 16 hr. at 38°. The water-soluble oxidation product was separated from the manganese dioxide and precipitated by addition of 1 vol. acetone at 0°. Recovery, 10·8 mg. P (90 %); inorganic P nil; ratio fatty acid ester/P, 1·01:1. This substance was hydrolysed in 2 N-HCl at 100° for 2 hr. and the fatty acids removed by filtration. The aqueous solution contained methyl glyoxal (Ariyama, 1928) and all the phosphorus and choline, but only traces of inorganic P (5·5 %) and free choline (7·0 %). Treatment with a phosphomonoesterase completely converted the P ester into inorganic phosphate and free choline, which showed that the P-containing product of the acid hydrolysis was phosphorylcholine.

The ethanolamine-based phosphatide fraction of heart muscle was degraded similarly. The end products were fatty acid, phosphorylethanolamine and methyl glyoxal.

The lysolecithin and lysokephalin formed by the removal of the aldehyde group from choline- and ethanolamine- plasmalogens are therefore the α-acyl isomers, giving on oxidation the α-acyl-β-keto derivatives; these on acid hydrolysis give fatty acids, phosphorylcholine or phosphorylethanolamine, and presumably dihydroxyacetone, which is converted into methyl glyoxal. On similar treatment the usual β-acyl lysolecithin gives fatty acids, phosphoglyceric acid and choline (Hanahan, 1954). The results show that in natural plasmalogens the aldehyde residue is exclusively linked to the β-carbon atom of the glycerol moiety.

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Nature of the Linkage of the Aldehyde Residue in Natural Plasmalogens. By HILDEGARD DEBUCH (introduced by M. G. MACFARLANE). (*Physiologisch-chemisches Institut der Universität, Köln, Germany*)

Having previously shown (Klenk & Debuch, 1954, 1955) that both choline and ethanolamine plasmalogens, from beef heart and human brain respectively, contain one fatty acid radical esterified with the glycerol, Debuch (1956) demonstrated that both plasmalogens gave the corresponding lysophospholipids after mild acid treatment. In 1954 the authors discussed three formulae, because nothing was then known about the linkage between the aldehyde and the glycerol. One of these formulae shows the aldehyde in the so-called enol form, giving in effect an ether bond with the glycerol.

In a compound with this formula, the double bond between the α- and β-C atoms of the aldehyde would be attacked by ozone. The final product after ozonization, hydrolysis and further oxidation of the enol ether of the hexadecanal would be a *n*-pentadecanoic acid and that of the octadecanal would be a *n*-heptadecanoic acid. The ester linkage of the plasmalogen would not be affected by ozone. However, all double bonds of the unsaturated aldehydes and acids would also be oxidized and a mixture of mono- and dicarboxylic acids would be formed. But these split products must have chain lengths of C_9 or less. Only from the saturated aldehydes would it be possible to get long chain fatty acids with an odd number of C atoms.

An ethanolamine–kephalin fraction from human brain, purified by countercurrent distribution, was ozonized and all double bonds finally split by further oxidation with peracetic acid, yielding carboxylic derivatives. These were separated into light petroleum-soluble material and water-soluble material respectively. The former fraction, which consisted of the monocarboxylic acids, was dissolved in acetone and cooled to −40°, when the longer chain acids crystallized out, leaving the short-chain acids in solution. After chromatography on Hyflo Supercel, two acids were recovered as main constituents. Paper chromatography showed that these had R_F values intermediate between C_{14} and C_{16}, and C_{16} and C_{18} *n*-fatty acids respectively. They correspond exactly to those of reference C_{15} and C_{17} acids obtained by synthesis.

Thus, it might be concluded that the aldehydes of the plasmalogens (acetalphospholipids) exist in the enol form.

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Tyrosine *O*-Sulphate as an Arylsulphatase Substrate. By K. S. DODGSON, F. A. ROSE and N. TUDBALL. (*Department of Biochemistry, University of Wales, Cardiff*)

Recently, it has been shown (Suzuki, Takahashi & Egami, 1957 and personal communication) that the sulphated polysaccharide of the mucous gland of *Charonia lampas* is sulphated by a system composed of an arylsulphate, an arylsulphatase, the carbohydrate acceptor and an unknown factor. If this type of system operates in other organisms it would seem feasible to assume the presence in living tissues of an arylsulphate whose specific role is that of a sulphate donor. Tyrosine *O*-sulphate, known to occur in at least one polypeptide (Bettelheim, 1954), might conceivably fill such a role. It was of interest therefore to investigate the ability of this compound to act as a substrate for arylsulphatase enzymes.

The arylsulphatases used were the type I enzymes (see Dodgson, 1956) from *Aspergillus oryzae*, *Alcaligenes metalcaligenes* and rat, human or ox livers (arylsulphatase C) and the type II enzymes from human liver (arylsulphatases A and B) and from *Patella vulgata*. The activities of these enzymes towards tyrosine *O*-sulphate were compared with those observed towards phenyl-, *p*-nitrophenyl- and 2-hydroxy-5-nitrophenyl sulphates.

As expected, the type II enzymes exhibited relatively low affinity and activity towards tyrosine *O*-sulphate, particularly arylsulphatase B which showed no activity over incubation periods up to 4 hr. On the other hand, the type I enzymes, with one exception, showed appreciable affinity and activity towards the substrate. The exception, arylsulphatase C, although active towards phenyl- and *p*-nitrophenyl sulphates, did not hydrolyse tyrosine *O*-sulphate, irrespective of the experimental conditions used. Indeed the activity of the enzyme towards *p*-nitrophenyl sulphate was

enhanced in the presence of tyrosine *O*-sulphate. Arylsulphatase C (irrespective of the source) is an insoluble microsomal enzyme (Dodgson, Rose & Spencer, 1957). Solubilization of the rat enzyme by cationic, anionic or non-ionic detergents did not affect the inertness of the enzyme towards the substrate.

The results show that, of the three human enzymes, only arylsulphatase A is active (weakly) towards tyrosine *O*-sulphate. However, there is some evidence (Glendenning, Greenberg & Fraenkel-Conrat, 1947) which suggests that rapid desulphation of the tyrosine residues of sulphated insulin can occur in the intact animal and experiments are now being conducted to test whether arylsulphatase C is active towards tyrosine *O*-sulphate when the amino and carboxyl groups of the latter are participating in peptide or other linkages.

One of us (N.T.) is grateful for an M.R.C. studentship.

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The Biological Conversion of Tetraethyltin to Triethyltin. By JILL E. CREMER. (*Toxicology Unit, M.R.C. Laboratories, Carshalton, Surrey*)

The symptoms of poisoning in rabbits and rats injected intravenously with tetraethyltin, after an initial latent period, closely resemble those of triethyltin. Stoner, Barnes & Duff (1955) suggested that tetraethyltin was converted *in vivo* to triethyltin.

By making use of the method for determining triethyltin based on the formation of a colour complex with dithiocarbazone (Aldridge & Cremer, 1957; Cremer, 1957) it has been possible to detect triethyltin in tissue samples from animals injected with tetraethyltin. The amount of triethyltin appearing in the tissues increased with time reaching a maximum between 1 and 2 hr. The *in vitro* metabolism of brain slices prepared from injected rats was inhibited and there was a direct correlation between the degree of inhibition and the amount of triethyltin present in the brain. The results were similar to those previously described for rats given triethyltin (Cremer, 1957).

In vitro experiments showed that the main site of conversion was in the liver. Liver slices were active in Krebs–Ringer–phosphate medium but whole homogenates required nicotinamide, $MgSO_4$ and triphosphopyridine nucleotide for maximum conversion activity. Simple cell-fractionation studies showed the microsome plus supernatant fraction was the most active.

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***In vitro* Studies of Isotope Incorporation from [^{14}C]Glucose into the Ribose of the Purine and Pyrimidine Nucleotide Components of Ribonucleic Acid.** By S. ITZHAKI (introduced by BARBARA E. HOLMES). (*Department of Radiotherapeutics, University of Cambridge*)

The turnover and biosynthesis of nucleic acids have been extensively studied with the use of ^{32}P inorganic phosphate and of several isotope-labelled precursors of the purine and pyrimidine bases. However, the sugar moieties of the nucleotides have received little attention. Relatively few studies have been carried out on the biosynthesis of ribose and even fewer on that of deoxyribose. Recent advances in the study of carbohydrate metabolism and the elucidation of the direct oxidative pathway of glucose in various biological systems show the importance of this pathway in cellular metabolism as a source of ribose (see review by Glock, 1955).

In the present study slices and cell suspensions were incubated aerobically in the presence of [U-^{14}C]glucose. At the end of incubation the tissues were fractionated according to the method of Schmidt & Thannhauser (1945) and the ribonucleic acid hydrolysed to mononucleotides.

In the first series of experiments the ribonucleotides were precipitated as their barium salts (Roll & Weliky, 1955). These were hydrolysed with N-HCl, the solution deionized, and the ribose isolated by

paper chromatography. The relative specific activity (RSA)* of the purine-ribose thus obtained from various tissues was in the following descending order: rat thymus, rat spleen, mouse Ehrlich ascites tumour, and rat liver. The thymus preparation also gave the most consistent results, the RSA varying between 1·30 and 1·80.

In the second series, both purine- and pyrimidine-bound ribose were isolated. The pyrimidine nucleotides were treated with bromine solution and alkali (Cohn, 1956) and hydrolysed with acid to liberate free ribose. It was found that the specific activity of the pyrimidine-ribose was 25–35% of that of the purine-ribose in the case of thymus, and about 40% in ascites tumour. No incorporation was noticed into the ribose of either purine or pyrimidine nucleotides when ascites tumour cells were incubated anaerobically.

When the four ribonucleotides were separated by paper electrophoresis (Markham & Smith, 1952) the ribose of adenylic and guanylic acids had the highest activity, while cytidylic acid ribose showed a low specific activity.

* RSA = specific activity of ribose (c.p.m./μmole carbon) $\times$ 100/specific activity of glucose (c.p.m./μmole carbon).

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Extraction and Fractionation of Steroid Conjugates. By I. E. Bush and Muriel Gale. (*The Radcliffe Infirmary, Oxford*)

There is no method for the estimation of urinary steroids as their conjugates which is convenient for routine use, although various methods exist for preparative work, for the estimation of conjugated pregnanediol, and for conjugated Porter–Silber chromogens (Reddy, 1954). A general method for extracting and fractionating urinary steroid conjugates would be useful, since the problems of hydrolysis of the conjugates would be avoided and other classes of conjugate than those known could be looked for.

After initial difficulties a satisfactory method was devised for extracting the major steroid conjugates of human urine, using the anion-exchange resin 'Decolorite' (Permutit Ltd.). 60–100 ml. urine at pH 3·0 are percolated through a 1 cm. $\times$ 20 cm. column of resin in chloride form (24–50 mesh) at 2–3 ml./min. followed by 40 ml. water. Conjugates are then eluted with 80 ml. methanol–ammonium hydroxide (9:1, v/v, 0·880 sp.gr. ammonia). Both free and conjugated steroids are recovered in this eluate. Good recoveries depend on exhaustive washing of the resin and careful removal of fines before use. Carefully washed resin-chloride is stable for at least 2 months at approx. 4°. Methanol is not the only suitable eluant but was chosen because of the obvious role of non-specific forces in the adsorption of steroids on resins. Thus Anderson & Warren (1951) removed all 17-oxosteroid conjugates from urine with a *cation*-exchanger but could not elute them with aqueous solutions.

The eluate from the resin is evaporated at 40–45° under reduced pressure and is suitable for enzymic hydrolysis, or further purification before chromatography of the conjugates themselves (Bush, 1957). The eluate is best dissolved in 5 ml. 1/5-saturated Na_2SO_4 at pH 2–3 and extracted once with 10 ml. *sec*-butanol or tetrahydrofurane followed by washing. This gives a small clean extract and better recovery of tetrahydrocortisol-glucuronoside than *n*-butanol. These solvents are far more selective for steroids than the ether–ethanol method (Edwards, Kellie & Wade, 1953) which was originally designed for conjugates of lower molecular weight by Kamil, Smith & Williams (1953). Separation of sulphates from glucuronosides is easily achieved by using the alkaline solvent systems described earlier (Bush, 1957).

We are grateful to Professor F. L. Warren for details of his earlier work with resins.

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Taurine Conjugates in Pig Bile Salts. By G. A. D. Haslewood and A. U. Ogan. (*Guy's Hospital Medical School, London, S.E.* 1)

Pig bile salts are largely glycine conjugates. In view of the unique chemical nature of the bile acids, it seemed of interest to investigate the taurine conjugates which also occur.

For separation, the two phases (*A*, 70%, v/v, acetic acid:water and *B*, 80%, v/v, 'amyl acetate': *n*-heptane) of the 'taurine system' of Sjövall (1955) were used. In this system, on paper, glycine conjugates move with the solvent ('amyl acetate': heptane) front.

Fresh bile salts (40 g.; S, 0·1%) in phase *A* (100 ml.) were washed with 10 portions of 100 ml. of phase *B*; *A* was evaporated and the residue (17·3 g.) in fresh *A* (100 ml.) was again washed 12 times as above. Evaporation of the *A* phase now gave a residue (10·6 g.) which in fresh *A* (50 ml.) was washed with 12 (50 ml.) portions of *B*. *A* was evaporated and the residue (8·6 g.) in fresh *A* (50 ml.) washed finally 30 times with 50 ml. portions of *B*.

The final product (2·3 g.) obtained by evaporation of the acetic acid phase (*A*) was a dark impure gum (S, 2·4%): it showed no glycine conjugates when run on paper in the 'glycine system', G_3, of Haslewood & Sjövall (1954).

In the above 'taurine system' on paper, the final product showed definite spots suggesting the presence of taurohyocholate and taurochenodeoxycholate. After hydrolysis and esterification (including acetylation) of the bile acids by the usual methods, the following acids (as derivatives) were detected by chemical means and by paper chromatography: hyocholic, hyodeoxycholic, chenodeoxycholic, 3α-hydroxy-6-oxo-*allo*cholanic. These are the main acids already known to be present in pig bile. The three non-ketonic acids have previously been shown, by chromatography, to occur as glycine conjugates (Haslewood & Sjövall, 1954); thus these substances are conjugated with both glycine and taurine.

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The Significance of a Second Macromolecular Component Observed in Preparations of *Clostridium botulinum* type A Toxin. By K. A. Cammack. (*Microbiological Research Establishment, Porton, Wiltshire*)

The isolation of *Cl. botulinum* type A toxin as a crystalline protein has been described by Lamanna, McElroy & Eklund (1946*a*, *b*) and Abrams, Kegeles & Hottle (1946). Putnam, Lamanna & Sharp (1946, 1948) confirmed its homogeneity in the ultracentrifuge and calculated a molecular weight of 900 000 using sedimentation and diffusion data obtained at pH 4·38. However, the sedimentation constant has been found to increase with pH (Wagman & Bateman, 1951), and it appears that the molecule exhibits maximum physical stability around pH 3·8. At pH 7·5 dissociation into toxic subunits has been reported by Wagman & Bateman (1953) and Wagman (1954); their work suggests that the toxic and haemagglutinating properties of the toxin are associated with different subunits within the native molecule, adding weight to the observations of Lamanna & Lowenthal (1951) who, with the aid of the Oudin (1946, 1952) serum–agar technique, showed that crystalline toxin produces two precipitin zones.

We have been investigating some type A toxin samples (specific toxicity = [170–210] × 10^6 LD 50/mg. N, 20 g. mouse, intraperitoneal route) which were prepared by a method based on a procedure of Bottomley & Creeth (unpublished work) and briefly described by Halliwell (1954). In the analytical ultracentrifuge at pH 3·8, a second well-defined macromolecular species, $S^0_{20,w} = 11{\cdot}9$, resolves from the toxin peak, $S^0_{20,w} = 16{\cdot}3$. Attempts to increase the differential sedimentation rate by working at higher pH values have failed, since we find that the sedimentation rates of both components increase with, and tend to converge with, rise in pH. At pH 4·27 a single, asymmetrical boundary replaces the two symmetrical boundaries observed at pH 3·8. The appearance of this boundary is markedly dependent upon ionic strength, and the formation at $I = 0{\cdot}2$ of an aggregate, $S_{20,w} \sim 40$, is of particular interest.

Immunologically our preparations are indistinguishable from the crystalline material of Lamanna & Lowenthal (1951) when analysed by the Ouchterlony (1948) double-diffusion technique. Fractions enriched with the $S^0_{20,w} = 11{\cdot}9$ component have been obtained using a partitioned ultracentrifuge cell and comparative specific toxicity determinations suggest that it is non-toxic. Attempts have been made to associate haemagglutinating activity with this component and these will be discussed.

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The Effect of Lysolecithin on Certain Enzymic Activities of Brain. By ELIZABETH A. MARPLES, R. H. S. THOMPSON and G. R. WEBSTER. (*Department of Chemical Pathology, Guy's Hospital Medical School, London, S.E.* 1)

Recent work in this laboratory (Webster, 1957) has shown that when lysolecithin is added to homogenates of rat brain, a very rapid and complete 'clearing' of the opaque homogenate occurs. Morrison & Zamecnik (1950) had earlier suggested that if lysolecithin were to be formed locally inside the central nervous system, localized demyelination might result. The effect of lysolecithin, prepared by the method of Long & Penny (1957) from egg lecithin, purified by chromatography on an alumina column according to the method of Rhodes & Lea (1957), and also the effect of the lysis produced by this compound, has been determined on certain of the enzymic activities of rat brain.

We have initially studied those enzymes which, if released into solution in an active and uncoordinated state, might be expected to exert a further destructive action on cells or cell membranes, or to interfere with the normal processes of excitation and transmission in unaffected nervous tissue.

True- and pseudo-cholinesterase activity have been found to be unaffected by 'clearing' the brain homogenates with lysolecithin. Tributyrinase activity, on the other hand, shows about 60% inactivation. Alkaline phosphatase in the 'cleared' homogenates shows a slight activation over the level in untreated homogenates, while the acid phosphatase is about 45% inactivated. Glycerylphosphorylcholine diesterase is unaffected, as are proteinase (cathepsin) and transaminase (glutamic-oxaloacetic) activity. Mono-amine oxidase (with tyramine as substrate, and in the presence of cyanide) shows about 30% inactivation in the 'cleared' preparations.

The addition of the lysolecithin to the brain homogenates is also followed by the production of acid. When this is measured manometrically, the rate of CO_2 evolution is of the order of 5000 μl./g. tissue/hr. for the first 5 min., after which it falls off rapidly to a steady evolution of about 600 μl./g./hr. which is maintained for at least 2 hr. although the 'clearing' process is complete in <60 min. The question arises whether this acid production is due to the action of a phospholipase B, the existence of which in brain has recently been claimed by Dawson (1956) on different evidence.

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A Fluorescent Reaction with Bile Acids. By M. D. TURNER, E. C. OSBORN and I. D. P. WOOTTON. (*Department of Chemical Pathology, Postgraduate Medical School, Ducane Road, London, W.* 12)

Numerous compounds of biological interest fluoresce under certain conditions. Duggan, Bowman, Brodie & Udenfriend (1957) have investigated a great number of compounds including polycyclic aromatic compounds. Pesez (1953) has shown that phosphoric acid acts like hydrochloric acid in the reaction suggested by Hammerstein in which heating of cholic acid in hydrochloric acid results in a blue fluorescence under ultraviolet light. This reaction is similar to the phospho–vanillic reaction. Sweat (1954) has shown that sulphuric acid-induced phenomena may be adapted to the quantitative

analysis of corticosterone and 17-hydroxycorticosterone by reacting ethanolic solutions of these steroids with sulphuric acid.

We have found that methyl esters of cholic acid, deoxycholic acid, chenodeoxycholic and lithocholic acid fluoresce when treated with concentrated sulphuric acid at room temperature. Preliminary experiments indicate that fluorescence increases slowly with time over a period of 24 hr. in the case of methyl deoxycholate. With the remaining three esters, development of fluorescence is more rapid, being most rapid in the case of methyl cholate.

Subsequent experiments have shown that the rate of development of fluorescence and also its degree can be greatly influenced by heat. Over a period of 1 hr. heating to 100° increases the fluorescence obtained by two to eight times over that obtained at room temperature, the actual increase varying with the ester. The effect is most pronounced in the case of methyl deoxycholate and least with methyl cholate. The exciting incident light lies in the range 455–465 mμ and the emitted light in the range 435–505 mμ.

Graphs constructed from data obtained at varying temperatures and concentrations indicate that within a certain range of concentrations, the degree of fluorescence is proportional to the concentration of the ester. Above a certain concentration the sensitivity is decreased because of 'quenching' effects. Interference may also arise from non-fluorescing coloration.

By making use of fluorescence it should be possible to measure absolute amounts of the bile esters of the order of 1–10 μg. to an accuracy of 0·5 μg.

Ultimately it is hoped to employ these methods in the analysis of bile acid conjugates recovered from blood making use, in the final stages, of paper chromatography or silica column partition to separate the various acids.

We must thank Dr V. H. T. James for help with some of the fluorescence measurements.

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DEMONSTRATIONS

Starch Gel Electrophoresis of Cattle Serum Proteins. By G. C. Ashton. (*Farm Livestock Research Station, Stock, Essex*)

By means of starch gel electrophoresis in boric buffer Smithies (1955) has demonstrated human serum protein differences which proved to be under genetic control. Similar differences were not obtained with other mammalian sera using boric buffer (Ashton, 1957), but with phosphate buffer genetically controlled serum protein differences have been found in cattle. These differences are of two sorts, those due to variation in the number and staining intensity of the β_2-globulins, of which four, five or six may be present in cattle, and those due to the presence or absence of one or both of a pair of components apparently derived from the albumin ('thread proteins'). Five phenotypes have been found due to differences in the β_2-globulin pattern and four phenotypes due to differences in the 'thread proteins'. The demonstration shows details of the technique, examples of the various phenotypes, results from about 100 matings, and the distribution of phenotypes with respect to breed.

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An Apparatus for Measuring Simultaneously the Oxygen Consumption and Contraction Response of an Electrically Stimulated Muscle Preparation. By A. Darbre and R. L. Speirs. (*Departments of Biochemistry and Physiology, King's College, Strand, London, W.C. 2*)

The apparatus, which is placed in a thermostatically-controlled water bath (±0·002°), consists of a main chamber holding about 10 ml. medium, side tubes, valve system and Warburg manometer. Oxygen is continuously bubbled through the medium and recirculated by means of a pumping system, which is entirely closed. The lower end of the muscle is held rigidly by one electrode in the main chamber, whilst the upper end is attached to a platinum wire (this forms the second electrode) which leads out through a mercury seal. The free end of the platinum wire is attached to a suitable

lever in order to record contractions. The oxygen consumption is obtained by the direct method of Warburg, carbon dioxide being absorbed as the gas passes over KOH. Manometric readings are taken at intervals when the pumping of the gas is momentarily stopped. The total gas volume of the system is about 5 ml. A side arm on the main chamber affords a means for readily obtaining samples for analysis and for changing the medium or altering its composition.

Resistance to Ketosis in the Obese Human on High-Fat Low-Calorie Diets. By G. L. S. PAWAN. (*Department of Medicine, Middlesex Hospital, London, W.* 1)

Eighteen obese female patients, 35–140% above their 'ideal' body weights, were given 90% fat, 1000 kcalorie diets daily, for periods of 5–14 days. Water and sodium chloride were kept constant at 3 l. and 10 g. daily. Rapid weight loss occurred in all patients. The following estimations were made at the beginning and end of the diet periods, on blood and urine. Glucose (King & Garner, 1947), plasma bicarbonate (Van Slyke manometric method; and Scribner & Caillouette, 1954) and 'ketone bodies' (Dumm & Shipley, 1946; Nadeau, 1952) after decarboxylation of acetoacetate in acid solution at 100° in sealed tubes. The latter method yielded good recoveries of added acetone and acetoacetate from blood and urine.

The high-fat metabolic mixture was well tolerated by the obese patients. Blood sugar was maintained within normal limits, ketonaemia was not marked nor was plasma bicarbonate much reduced. Some ketonuria occurred but it was never severe.

In contrast, one apparently normal non-obese subject developed hypoglycaemia and severe ketosis after 3 days on this diet. In two other non-obese normal subjects, an 80% fat diet supplying about 1500 kcalories daily also produced ketosis and the diets had to be terminated after 4 days.

Resistance to ketosis in the very obese may be the result of enzymic adaptation affecting metabolic pathways of fat catabolism.

I am grateful to Professor Alan Kekwick for encouragement with this work.

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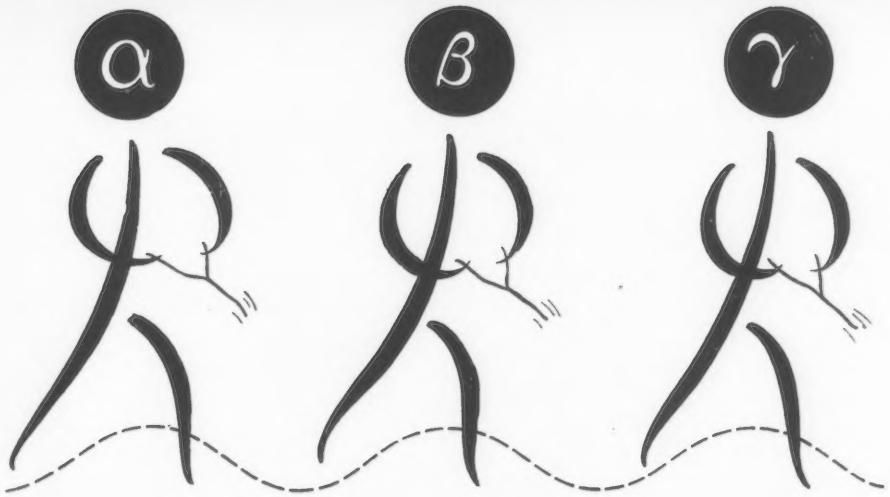
Apparatus for Ion-Exchange Chromatography. By R. W. R. BAKER. (*Department of Chemical Pathology, Guy's Hospital Medical School, London, S.E.* 1)

A column of Zeo-Karb 225 in micro-bead form has been used for resolving amino acids and other substances extracted from nerve tissue. The column and an ancillary apparatus for changing gradually the composition of the eluant are shown, together with some of the results that have been obtained.



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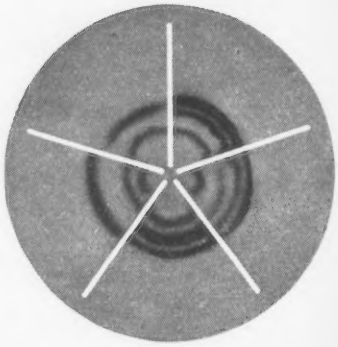

This important addition to chromatographic instrumentation has numerous applications in all fields of chemistry. It has proved extremely useful in the routine analysis of urine for sugar (e.g., the separation of galactose in the presence of glucose is effected by a four-hour run) and the screening of large numbers of samples in surveys dealing e.g. with the distribution of amino acids. In industrial laboratories the apparatus can be used for rapid semi-quantitative analysis to check manufacturing processes. The small 14 cm. outfit is ideal for teaching chromatography in schools and colleges.

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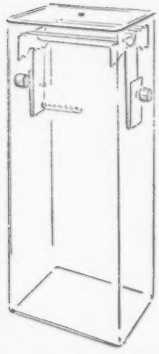

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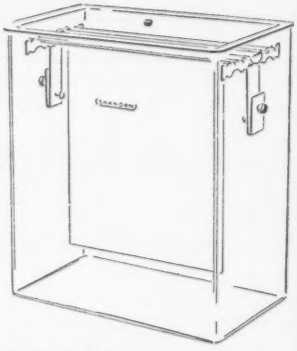

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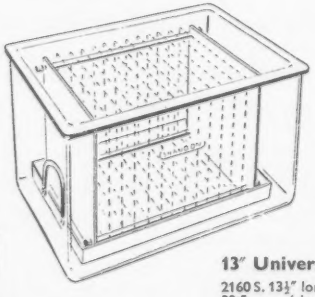

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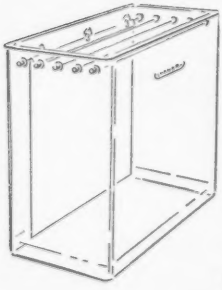

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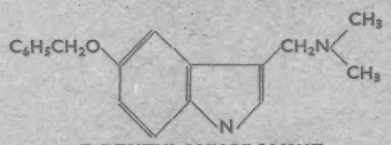
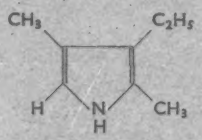
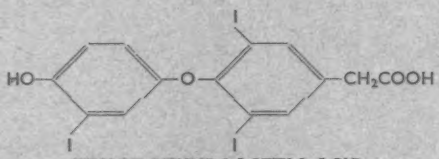


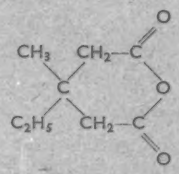
5-BENZYLOXYGRAMINE



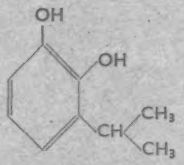
KRYPTOPYRROLE



TRIIODOTHYROACETIC ACID
(Diethanolamine Salt)
DIIODOTHYROACETIC ACID also available



β-METHYL-β-ETHYLGLUTARIC ANHYDRIDE



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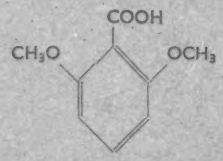

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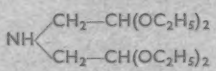

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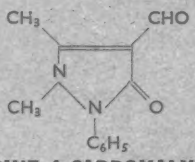

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